

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
23 June 2005 (23.06.2005)

PCT

(10) International Publication Number  
**WO 2005/055920 A2**

(51) International Patent Classification<sup>7</sup>: **A61K**

(21) International Application Number:  
PCT/IL2004/001117

(22) International Filing Date: 9 December 2004 (09.12.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/527,772 9 December 2003 (09.12.2003) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITIONS AND METHODS FOR TREATMENT OF PSYCHIATRIC DISORDERS

(57) Abstract: An agent selected from: (i) dopamine; (ii) a dopamine precursor; (iii) a dopamine D1-R agonist; (iv) a combination of dopamine and a dopamine precursor; (v) a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with a dopamine D2-R antagonist; (vi) a modified CNS-peptide; (vii) T cells that have been activated either by a CNS-specific antigen or by a modified CNS-peptide; and (viii) poly-YE, a poly-YE related peptide or polypeptide, can cause down-regulation of the suppressive activity of CD4+CD25+ regulatory T (Treg) cells on CD4+CD25- effector T cells (Teff), modulation of the immune response and/or modulation of autoimmune response, and are useful for treatment of psychiatric disorders.



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## COMPOSITIONS AND METHODS FOR TREATMENT OF PSYCHIATRIC DISORDERS

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### FIELD OF THE INVENTION

The present invention relates to compositions and methods for treatment of psychiatric disorders and, in particular, to such a treatment with an agent that causes  
10 down-regulation of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells on CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (Teff), modulation of the immune response and/or modulation of autoimmune response.

**Abbreviations:** **APCs:** antigen-presenting cells; **APL:** altered peptide  
15 ligand; **ASR:** acoustic startle response; **BSA:** bovine serum albumin; **CBC:** cut-off behavioral criteria; **CNS:** central nervous system; **CSPG:** chondroitin sulfate proteoglycans; **CTLA-4:** cytotoxic T-lymphocyte-associated antigen receptor 4; **D-R:** a dopamine receptor **D1-R:** dopamine receptor type 1; **D2-R:** dopamine receptor type 2; **DA:** dopamine; **EAE:** experimental autoimmune encephalomyelitis; **EPM:**  
20 Elevated plus-maze; **ERK:** extracellular signal-regulated kinase; **MAG:** myelin-associated glycoprotein; **MBP:** myelin basic protein; **MDC:** macrophage-derived chemokine; **MOG:** myelin oligodendrocyte glycoprotein; **mrIL-2:** mouse recombinant interleukin-2; **MWM:** Morris water maze; **NE:** norepinephrine; **NS:** nervous system; **PCR:** polymerase chain reaction; **PLP:** proteolipid protein; **PE:**  
25 phycoerythrin; **PNS:** peripheral nervous system; **poly-YE:** poly-Tyr,Glu; **PTSD:** post-traumatic stress disorder; **RGCs:** retinal ganglion cells; **SCID:** severe combined immune deficiency; **SDF-1:** stromal cell-derived factor-1; **TBI:** total body  $\gamma$ - irradiation; **Teff:** effector T cells; **TLI:** total lymphoid organ  $\gamma$ -irradiation; **Treg:** regulatory T cells; **WT:** wild-type.

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## BACKGROUND OF THE INVENTION

### Psychiatric or mental disorders

The causes of psychiatric or mental disorders are complex. Genetic, biological, and environmental factors can contribute to their development. For many disorders, the causes have not yet been determined. For many of these disorders, it appears that they occur as a result of abnormalities in the levels of certain neurotransmitters in the brain, such as glutamate toxicity.

Among the psychiatric or mental disorders are the: (i) anxiety disorders, that include phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder, schizophreniform disorder, schizoaffective disorder and delusional disorder; and (iv) drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, phencyclidine use, etc. (The Merck Manual, Section 15, 17<sup>th</sup> Edition, 1999, Merck Research Laboratories, Whitehouse Station, N.J., U.S.A.).

Schizophrenia is a common and serious mental disorder which worldwide prevalence appears to be 1%, although pockets of higher and lower prevalence exist in some countries. The cause of schizophrenia has not yet been determined, although research points to the interaction of genetic endowment and major environmental upheaval during development of the brain. In the research of schizophrenia, two lines of research are beginning to converge: neurodevelopmental disruption may be the result of genetic and/or environmental stressors early in development, leading to subtle alterations in the brain. Furthermore, environmental factors later in development can either exacerbate or ameliorate expression of genetic or neurodevelopmental defects. The overarching message is that the onset and course of schizophrenia are most likely the result of an interaction between genetic and environmental influences.

Current research proposes that schizophrenia is caused by a genetic vulnerability coupled with environmental and psychosocial stressors, the so-called diathesis-stress model. Family studies suggest that people have varying levels of inherited genetic vulnerability, from very low to very high, to schizophrenia.

5 Whether or not the person develops schizophrenia is partly determined by this vulnerability. At the same time, the development of schizophrenia also depends on the amount and types of stresses the person experiences over time.

Excessive levels of the neurotransmitter dopamine have long been implicated in schizophrenia, although it is unclear whether the excess is a primary cause of

10 schizophrenia or a result of a more fundamental dysfunction. More recent evidence implicates much greater complexity in the dysregulation of dopamine and other neurotransmitter systems. Some of this research ties schizophrenia to certain variations in dopamine receptors, while other research focuses on the serotonin system.

15 Almost all the neurons in the brain are influenced by the excitatory amino acid glutamate. Glutamatergic neurotransmission has been associated functionally with a number of physiological processes and with certain pathophysiological processes, including schizophrenia. Imaging studies provide indirect evidence that glutamate may be involved in schizophrenia.

20 Pharmacotherapies are the most extensively evaluated intervention for schizophrenia. The conventional or older antipsychotic medications (e.g., chlorpromazine, haloperidol, fluphenazine, molindone) and the more recently developed medications (e.g., clozapine, risperidone, olanzapine, quetiapine, sertindole) are used to reduce the positive symptoms of schizophrenia. The newer

25 medications, often called atypical because they have a different mechanism action than their predecessors, also appear in preliminary studies to be more effective against negative symptoms, display fewer side effects, and show promise for treating people for whom older medications are ineffective. Their introduction has created more treatment options for people with schizophrenia and other serious

30 mental illnesses. Although the newer, more broadly effective medications have

increased hopes for recovery, they also have resulted in greater treatment complexity for patients and providers alike. Efficacy data on the newer antipsychotics indicate that they are as efficacious as the older agents at reducing positive symptoms and carry fewer side effects. However, the use of the new drug  
5 clozapine was constrained for many years in the United States because of findings that in about 1 percent of patients it causes a potentially fatal blood condition: agranulocytosis, a loss of white blood cells that fight infection. Although effective safeguards exist, use of clozapine tends to be limited to those who are unresponsive to, or cannot tolerate, other antipsychotics.

10 Post-traumatic stress disorder (PTSD) is an anxiety disorder that can develop after exposure to a terrifying event or ordeal in which grave physical harm occurred or was threatened. Traumatic events that can trigger PTSD include violent personal assaults such as rape or mugging, natural or human-caused disasters, accidents, or military combat. PTSD can be extremely disabling. Research has demonstrated the  
15 effectiveness of cognitive-behavioral therapy, group therapy, and exposure therapy, in which the patient gradually and repeatedly relives the frightening experience under controlled conditions to help him or her work through the trauma. Studies have also shown that medications help ease associated symptoms of depression and anxiety and help promote sleep. Scientists are attempting to determine which  
20 treatments work best for which type of trauma.

#### **CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg)**

The naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> cells, which comprise about 10% of the total CD4<sup>+</sup> population, are the so-called regulatory T cells (hereinafter designated  
25 “Treg”), originally called suppressor T cells. These cells express the transmembrane protein called CD25, the  $\alpha$  chain of the IL-2 receptor (Sakaguchi et al., 1995).

The antigenic peptides recognized by the T-cell receptors of Treg tend to be self-peptides and, perhaps, the major function of Treg cells is to inhibit other T cells (effector T cells, hereinafter “Teff”) from mounting an immune attack against self  
30 components, namely, to protect the body against autoimmunity. Indeed, it has been

confirmed that naturally occurring Treg suppress autoimmunity (Shevach et al., 2001; Sakaguchi et al., 1995).

As described below, recent evidence provided by the present inventors indicate that autoimmunity, that has long been viewed as a destructive process, is the body's endogenous response to central nervous system (CNS) injury and its purpose is in fact beneficial (Schwartz and Kipnis, 2001; Yoles et al., 2001). This neuroprotective autoimmunity was shown by the inventors to be inhibited by naturally occurring  $CD4^+CD25^+$  cells, that suppressed an endogenous T-cell mediated neuroprotective mechanism to achieve maximal activation of autoimmunity and, therefore, to withstand injury to the CNS (Kipnis et al., 2002a).

Treg-imposed suppression is a multifactorial process, involving cell-to-cell contacts (Nakamura et al., 2001) and the activity of soluble factors [which presumably include IL-10 (Sundstedt et al., 2003) and TGF- $\beta$  (Piccirillo et al., 2002)]. Studies have shown that the suppressive activity of Treg can be inhibited by the addition of exogenous IL-2 (Thornton and Shevach, 1998), or blocking of the cytotoxic T-lymphocyte-associated antigen receptor 4 (CTLA-4) (Nakamura et al., 2001), or activation of the newly discovered glucocorticoid-induced TNF- $\alpha$  receptor (McHugh et al., 2002).

Some key adhesion molecules are more abundant on the surfaces of Treg than of Teff (Kohm et al., 2002). The ability of Treg to enter tissues might help prevent autoimmune disease progression. In fighting off neurodegeneration or cancer, however, the presence of Treg is a liability. Compounds capable of reducing the trafficking ability (adhesion and migration) of Treg, or their suppressive activity, or both, might therefore be promising candidates for therapy against both cancer and CNS insults. As a corollary, compounds capable of upregulating the inhibitory or trafficking activity of Treg, or both, might be potential candidates for therapy against autoimmune diseases. A fine balance would then be needed to fight off the conditions leading to neuronal degeneration without creating conditions that foster neural tissue-specific autoimmune diseases. Up to now, however, no physiological compounds have been discovered that can control the activity of Treg.

**Protective autoimmunity**

Injury to the CNS triggers the immediate death of injured neurons, and this is inevitably followed by a series of destructive processes, collectively termed  
5 secondary degeneration (Yoles and Schwartz, 1998), which result in the gradually spreading degeneration and death of initially undamaged adjacent neural cells. The processes of secondary degeneration are mediated mainly by destructive self-compounds that emanate from the directly damaged neurons and render the extracellular environment hostile to recovery. Until very recently, the prevailing  
10 view was that the CNS, being an immune-privileged site, cannot benefit from immune intervention and that all immune activity is detrimental. Studies by our group showed, however, that one way in which the hostility of the environment at the damaged site can be circumvented is by eliciting a systemic defensive activity that homes to the lesion site and helps the innate arm of the immune system to fight  
15 off the toxicity. This assistance is provided by the spontaneous recruitment of T cells specific to CNS-related self-antigens (Kipnis et al., 2002a). The autoimmune T cells home to the site of the lesion and become activated there by encountering their specific antigens, which are presented to them by antigen-presenting cells (e.g. activated microglia). Thus, contrary to the prevailing belief that the immune system  
20 is always harmful to the CNS, our work suggested that the CNS withstands injurious post-injury conditions by eliciting a protective autoimmunity (Moalem et al., 1999).

Further studies by our group showed that adult rats or mice deficient in mature T cells, or deprived (as a result of immunization at birth with spinal cord  
25 homogenate) of T cells specific to self-antigens residing in the site of damage, are unable to withstand injurious conditions in the CNS (Kipnis et al., 2001; Schori et al., 2002). The T cells that participate in protection were found to possess a phenotype characteristic of Th1 cells (Kipnis et al., 2002b). These and related results led us to formulate the concept of “protective autoimmunity” as a  
30 physiological mechanism of protection against destructive self-compounds

(Schwartz and Kipnis, 2001). Our group showed that this physiological response can be boosted by injection (passive transfer) of activated autoimmune T cells (Moalem et al., 1999; Kipnis et al., 2002b; Hauben et al., 2000b) or by active vaccination with self- or self-related antigens (Hauben et al., 2000a, 2001a, 2001b; Schori et al., 2001). Moreover, the spontaneous protective response was found to be suppressed by the constitutive presence of naturally occurring Treg. Thus, nude mice replenished with splenocytes deprived of Treg are better able to withstand injurious conditions in the CNS than their matched wild-type controls or nude mice replenished with a population consisting of the full complement of spleen cells (Kipnis et al., 2002a; Schwartz and Kipnis, 2002).

### **Low-dose $\gamma$ -irradiation**

Low-dose whole-body  $\gamma$ -irradiation decreases the lymphocyte pool, causing homeostasis-driven proliferation of lymphocytes (Safwat, 2000; Ge et al., 2002). This proliferation restores the memory T cell compartment, producing more clones reactive to self-antigens; naïve T cells are reconstituted by new arrivals from the thymus. The cell proliferation is an outcome of cytokine stimulation and interaction between T-cell receptors, class II major histocompatibility complex proteins (MHC-II), and self-peptides. The proliferating cells acquire a memory phenotype and become hypersensitive to antigen stimulation. Simulation of lymphopenic conditions *in vitro* inhibits the homeostasis-driven proliferation of cells in co-culture with Treg (Goldrath et al., 2000; Cho et al., 2000).

### **Dopamine**

It is becoming increasingly clear that the body, to protect itself against tumor growth or CNS neurodegeneration, needs to elicit autoimmunity against self-antigens associated with the tumor (Dummer et al., 2002) or self-antigens residing in the site of degeneration (Moalem et al., 1999; Mizrahi et al., 2002; Schori et al., 2001), respectively. Normally, autoimmunity is suppressed by naturally occurring Treg (Shevach et al., 2001; Sakaguchi et al., 1995). Depletion of Treg enhances

neuronal survival after CNS insults (Kipnis et al., 2002) and increases spontaneous anti-tumor autoimmunity (Sakaguchi et al., 2001a, 2001b). Therefore, to elicit the desired autoimmune response for anti-tumor therapy or protection of CNS neurons at risk of degeneration, the Treg-imposed suppression must be blocked (Kipnis et al., 2002a; Schwartz and Kipnis, 2002). How this is achieved physiologically is not known.

Dopamine (3,4-dihydroxyphenylethylamine or 3-hydroxytyramine) is a catecholamine formed in the body by the decarboxylation of dopa (3,4-dihydroxyphenylalanine) and acts as a neurotransmitter in the CNS. It is used in all areas of the brain and is particularly important for regulating the function of the basal ganglia.

Parkinson's disease is a progressive degenerative disease caused principally by the degeneration of the dopaminergic cells in the substantia nigra pars compacta, with consequent loss of dopamine terminals in the striatum. The most widely used treatment for Parkinson's disease is pharmacotherapy, mainly by dopamine replacement because if dopamine is taken by mouth, it is rapidly degraded in the intestine and blood and it does not penetrate from the blood into the brain. Therefore, the precursor L-dopa (levodopa) is administered, instead of dopamine. L-dopa is converted to dopamine in the blood and in the brain. In order to maximize the effectiveness of L-dopa, it is usually given in combination with a medicine such as carbidopa, which blocks the conversion of L-dopa to dopamine in the blood. Therefore, more L-dopa is transported into the brain, where it is converted to dopamine.

Due to the side effects of the treatment with L-dopa or with the combination L-dopa/carbidopa, dopamine agonists have been developed or are in development for the treatment of Parkinson's disease and other diseases or conditions in which dopamine is involved. Contrary to levodopa, that is converted to dopamine in the body, the dopamine agonists mimic the activity of dopamine by directly activating the dopamine receptor rather than replace it as levodopa does.

The receptors for dopamine are primarily found in the striatum. There are at least five subtypes of dopamine receptors, called D1 through D5; the D1 and D5 subtypes belong to the dopamine receptor type 1 family and are referred to as "D1-like" or "D1-R" while the D2, D3, and D4 belong to the dopamine receptor type 2 family and are referred to as "D2-like" or "D2-R". The receptors are grouped in this manner because of the common properties of the receptor effects.

The different dopamine agonists may have affinity to both D1 and D2 families, albeit with different strength, or they may be specific to the D1 or the D2 family or to one of the receptors within one of the families. Thus, dopamine agonists having varying activities at the different dopamine receptors are known, or being investigated, that exhibit subtly different effects. Some of the dopamine agonists in use for treatment of Parkinson's disease include apomorphine (D1 and D2 agonist), the ergoline derivatives bromocriptine (D2 agonist), lisuride (D1 slightly partial agonist, D2 agonist), pergolide (D1 weak agonist, D2 and D3 strong agonist), and cabergoline (D2 agonist), and the non-ergoline derivatives ropinirole (D2 agonist) and pramipexole (D2 and D3 agonist). Other dopamine agonists under investigation include the D1 agonists dihydrexidine (DHX, the first high affinity full D1 dopamine receptor agonist), SKF-38393, SKF-81297, and SKF-82958, and the D2 agonists quinpirole, LY 172555, PPHT and quinlorane.

Besides their use in the treatment of Parkinson's disease, some dopamine agonists have been proposed for different indications. Bromocriptine and lisuride suppress prolactin secretion and can be used as prolactin inhibitor and in the treatment of prolactinomas. Bromocriptine and cabergoline lower serum growth hormone levels in acromegaly patients and can be used for treatment of acromegaly. US Patent No. 5,744,476 discloses the D1-R agonist dihydrexidine either alone or together with levodopa or with a D2-R agonist, for raising extracellular brain acetylcholine levels to improve cognition in a human having senile or presenile dementia associated with neurodegeneration.

Dopamine antagonists have been developed for several indications, particularly D2 antagonists such as sulpride, spiperone, haloperidol, spiroperidol, clozapine, olanzapine and sertindole for use as antipsychotic agents.

PCT/IL2004/000442 of the same applicant, filed on May 23, 2004, discloses  
5 a method for treating a neurodegenerative condition, disorder or disease other than Parkinson's disease, comprising administration of an agent that down-regulates the suppressive activity of Treg on Teff, in an amount effective to protect said individual from neurotoxic conditions, wherein said agent is selected from the group consisting of: (i) dopamine; (ii) a dopamine precursor; (iii) a D1-R agonist;  
10 (iv) a D2-R antagonist; (v) a combination of (i) and (ii); and (vi) a combination of (i), (ii) or (iii) with (iv).

PCT/IL2004/000441 of the same applicant, filed on May 23, 2004, discloses a method for modulating the suppressive effect of Treg on Teff, which comprises administration of dopamine, a dopamine precursor, a dopamine agonist, a dopamine  
15 antagonist, or a combination thereof. The method includes down-regulating the suppressive effect of Treg on Teff, for example for treatment of cancer (but excluding treatment of a neurodegenerative condition, disorder or disease) by administering an agent selected from the group consisting of: (i) dopamine; (ii) a dopamine precursor; (iii) a D1-R agonist; (iv) a D2-R antagonist; (v) a combination  
20 of (i) and (ii); and (vi) a combination of (i), (ii) or (iii) with (iv). The method also includes up-regulating the suppressive effect of Treg on Teff, for example for treatment of cancer or for control of graft rejection, by administering: (i) a D1-R antagonist; (ii) a D2-R agonist; and (iii) a combination of (i) and (ii).

## 25 **Poly-YE**

Poly-YE or poly-Glu,Tyr is a non-pathogenic synthetic random copolymer composed of the two amino acids L-glutamic acid (Glu, E) and L-tyrosine (Tyr, Y) in different proportions, for example, the copolymer poly-Glu<sup>50</sup>Tyr<sup>50</sup> with an average length of 100 amino acids and a capacity to elicit strong immune response  
30 in certain mouse strains. Poly-YE was described in WO 03/002140 of the present

applicant for preventing or inhibiting neuronal degeneration or for promoting nerve regeneration in the CNS or PNS, or for protecting CNS or PNS cells from glutamate toxicity.

## 5 **Modified CNS peptides and T cells activated thereby**

Activated T cells have been shown to enter the CNS parenchyma, irrespective of their antigen specificity, but only T cells capable of reacting with a CNS antigen seem to persist there. T cells reactive to antigens of the CNS white matter, such as myelin basic protein (MBP), can induce the paralytic disease  
10 experimental autoimmune encephalomyelitis (EAE) and anti-MBP T cells may also be involved in the human disease multiple sclerosis. However, despite their pathogenic potential, anti-MBP T cell clones are present in the immune systems of healthy subjects. Activated T cells, which normally patrol the intact CNS, transiently accumulate at sites of CNS white matter lesions (Hirschberg et al.,  
15 1998).

The present inventors discovered recently that activated T cells that recognize an antigen of the nervous system (NS) of the patient confer neuroprotection, as described in PCT Publications WO 99/60021 and WO 03/002602. More specifically, T cells reactive to MBP were shown to be  
20 neuroprotective in rat models of partially crushed optic nerve (see also Moalem et al, 1999) and of spinal cord injury (see also Hauben et al, 2000b). Before this discovery, it had been thought that immune cells do not participate in NS repair. Furthermore, any immune activity in the context of CNS damage was traditionally considered detrimental for recovery. It was quite surprising to discover that NS-  
25 specific activated T cells could be used to protect nervous system tissue from secondary degeneration which may follow damage caused by injury or disease of the CNS or peripheral nervous system (PNS). The massive accumulation of exogenously administered T cells at the site of CNS injury suggests that the presence of T cells at the site of injury plays a prominent role in neuroprotection.

In addition to the NS-specific activated T cells, the above-referenced PCT publications WO 99/60021 and WO 03/002602 disclose that therapy for amelioration of effects of injury or disease of the CNS or PNS can be carried out also with a natural or synthetic NS-specific antigen such as myelin-associated glycoprotein (MAG), S-100,  $\beta$ -amyloid, Thy-1, P0, P2, a neurotransmitter receptor, and preferably human MBP, human proteolipid protein (PLP), human oligodendrocyte glycoprotein (MOG), and Nogo, or with a peptide derived from said antigen.

In seeking a way to convert the experimental immunization into an effective post-traumatic therapy, our group has tested peptides that are derived from or cross-react with self-proteins and are “safe”, i.e., do not induce autoimmune disease. (Hauben et al., 2001a, 2001b). Among these peptides are peptides designated “altered peptides” or “altered peptide ligands” (APL), that are obtained by modification of a self-peptide derived from a CNS-specific antigen, which modification consists in the replacement of one or more amino acid residues of the self-peptide by different amino acid residues, said modified CNS peptide still being capable of recognizing the T-cell receptor recognized by the self-peptide but with less affinity (“modified CNS peptide”), particularly modified MBP-derived peptides, as described in WO 02/055010, and the modified Nogo and Nogo receptor-derived peptides as described in WO 03/002602.

Reference is made to copending international application entitled “Method and Vaccine comprising Copolymer 1 for treatment of psychiatric disorders” filed by applicant at the Israel Patent Office/ Receiving Office on the same date, the contents thereof being explicitly excluded from the scope of the present invention.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

## SUMMARY OF THE INVENTION

We have now found that protection against consequences of psychological trauma is T-cell dependent and is suppressed by naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells and, therefore, down-regulation of Treg and/or modulation  
5 of the immune response and/or modulation of the autoimmune response in an individual can be beneficial and can improve the individual's ability to withstand and cope with stressful conditions.

The present invention provides a method for treatment of an individual suffering from a psychiatric disorder which comprises administering to said  
10 individual in need of such a treatment an effective amount of an agent that causes down-regulation of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells on CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (Teff), modulation of the immune response and/or modulation of autoimmune response, but excluding Copolymer 1, a Copolymer 1-related peptide and Copolymer 1-related polypeptide, and further excluding a  
15 combination of dopamine, a dopamine precursor or an agonist of the dopamine receptor type 1 family (D1-R agonist) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist), when said individual is one suffering from schizophrenia.

In one embodiment, the agent that causes down-regulation of the suppressive  
20 activity of Treg on Teff is selected from the group consisting of (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) a combination of (i) and (ii); and (v) a combination of (i), (ii) or (iii) with an  
25 antagonist of the dopamine receptor type 2 family (D2-R antagonist) or a pharmaceutically acceptable salt thereof, provided that when said agent is a combination of (i), (ii) or (iii) with a dopamine D2-R antagonist, said individual is not one suffering from schizophrenia.

In another embodiment, the agent that causes down-regulation of the Treg cells is low dose whole-body or total lymphoid organ  $\gamma$ -irradiation that causes specific killing of Treg.

In a further embodiment, the agent is one that causes modulation of the autoimmune response and is, for example, a modified CNS-peptide obtained by  
5 modification of a self-peptide derived from a CNS-specific antigen, which modification consists in the replacement of one or more amino acid residues of the self-peptide by different amino acid residues, said modified CNS peptide still being capable of recognizing the T-cell receptor recognized by the self-peptide but with  
10 less affinity, or the agent is T cells that have been activated either by a CNS-specific antigen such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors, or by a modified peptide obtained by modification of a self-peptide derived from said  
15 CNS-specific antigen.

In yet a further embodiment, the agent is the copolymer poly-YE or a poly-YE related peptide or polypeptide, that cause down-regulation of the suppressive activity of the Treg cells, modulation of the immune response and modulation of autoimmune response. Poly-YE may be used as a vaccine for immunization of the  
20 individual in need.

The invention further provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and an agent as described above for treatment of psychiatric disorders. When said agent is poly-YE, the pharmaceutical composition may be a vaccine for immunization of the individual.

25 The invention still further provides the use of an agent as described above for the manufacture of a pharmaceutical composition for treatment of psychiatric disorders.

The invention yet further provides an article of manufacture comprising packaging material and a pharmaceutical composition contained within the  
30 packaging material, said pharmaceutical composition comprising an agent as

defined above; and said packaging material includes a label that indicates that said agent is therapeutically effective for treating a psychiatric disorder.

Examples of psychiatric or mental disorders that can be treated according to the invention include, but are not limited to: (i) anxiety disorders, that include  
 5 phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder, schizophreniform disorder, schizoaffective disorder and delusional disorder; (iv)  
 10 drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, and phencyclidine use; and (v) memory loss disorders such as amnesia or memory loss associated with Alzheimer's type dementia or with non-Alzheimer's type dementia, e.g. multi-infarct dementia or memory loss associated with Parkinson's disease,  
 15 Huntington's disease, Creutzfeld-Jakob disease, head trauma, HIV infection, hypothyroidism and vitamin B12 deficiency.

In preferred embodiments, the psychiatric disorder is schizophrenia, an anxiety disorder such as stress or post-traumatic stress disorder, or a mood disorder such as depression or a bipolar disorder.

20

## BRIEF DESCRIPTION OF THE FIGURES

**Figs. 1a-1c** show that the ability to withstand psychological stress depends on peripheral immunity. A single 10-min exposure to the odor of a predator caused behavioral changes in the examined mice. (1a-1c) In the BALB/c strain,  
 25 maladaptation was significantly more prevalent in SCID mice (61.9%) than in the wild-type (WT) mice (17.2%;  $\chi^2=10.6$ ,  $P<0.001$ ). Maladaptation was similarly more prevalent in nude mice (devoid of mature T cells only) than in the WT (70% and 17.2%, respectively;  $\chi^2=13.9$ ,  $P<0.0002$ ), verifying that the observed differences were attributable to the absence of mature effector T cells. (1b) SCID

and nude mice spent significantly more time in the closed arms of the elevated plus-maze (EPM) than did WT mice. \*\* -  $P < 0.01$ , between indicated groups. (1c) The acoustic startle response (ASR) (mean  $\pm$  SD) of SCID and nude mice was significantly higher than that of WT mice. \*\*\* -  $P < 0.01$ .

5       **Figs. 2a-2d** show that the ability to withstand psychological stress can benefit from autoimmune T cells. (2a) A single 10-min exposure to the odor of a predator resulted in maladaptation in about 30% of WT C57BL/6J mice and of  $T_{MBP}$  transgenic mice on the same background. More than 60% of  $RAG^{-/-}$  (or SCID) mice developed maladaptation to stress, whereas  $T_{MBP}/Rag1^{-/-}$  mice did not show any  
10 maladaptation. (2b)  $T_{MBP}/RAG^{-/-}$  mice spent significantly less time exploring the closed arms of the EPM than did other mice groups. \* -  $P < 0.05$ , between indicated groups. (2c) The ASR (mean  $\pm$  SD) of SCID mice was significantly higher than that of WT mice, however, surprisingly,  $T_{MBP}/RAG^{-/-}$  mice showed the highest ASR among the groups. The fact that the mean startle amplitude was higher in the  
15  $T_{MBP}/RAG^{-/-}$  mice indicates that these mice are more anxious than the other mice. But when evaluating the habituation for each group (exponential decay of the magnitude of the startle response during repeated acoustic stimulation), the results revealed otherwise. \* -  $P < 0.05$  vs. SCID,  $T_{MBP}$  and  $T_{MBP}/RAG^{-/-}$  groups; \*\* -  $P < 0.01$  vs. WT and SCID groups. (2d) The habituation curve of repeated acoustic  
20 startle stimuli.  $T_{MBP}/RAG^{-/-}$  mice exhibited the greater habituation of startle response (sharper slope) than WT, SCID and  $T_{MBP}$  mice, however in these mice the startle amplitudes decreased rapidly during the repeated startle stimuli.

**Figs. 3a-3c** show that naturally occurring Treg suppress the ability to withstand psychological stress. (3a) A single 10-min exposure to the odor of a  
25 predator resulted in maladaptation in 70% of nude male BALB/c mice (see Fig. 1). The prevalence of maladaptation was somewhat decreased (50%) in nude mice that were replenished with normal splenocytes from WT BALB/c mice. In nude mice that were replenished with a splenocyte population devoid of Treg, the prevalence of maladaptation (20%) was significantly lower than that of control  
30 (nonreplenished) nude mice ( $\chi^2=6.7$ ,  $P<0.009$ ). (3b) The ASR (mean  $\pm$  SD) of nude

mice replenished with splenocytes devoid of Treg was significantly weaker than that of nude mice replenished with a normal splenocyte population ( $P < 0.03$ ) or of control nude mice ( $F(df=2,37)=9.2$ ,  $P < 0.0006$ ). (3c) Nude mice replenished with splenocytes devoid of Treg spent significantly less time exploring the closed arms of the EPM than did nude mice replenished with a normal splenocyte population ( $P < 0.02$ ) or control nude mice ( $F(df=2,37)=8.7$ ,  $P < 0.0008$ ).

**Figs. 4a-4c** are micrographs showing that immunohistochemistry of T cells in the brain is correlated with adaptation to psychological stress. Maladapted animals from the group of nude mice replenished with a normal splenocyte population from WT mice and well-adapted animals from the group of nude mice replenished with splenocytes from WT mice devoid of Treg were killed and their brains were removed, perfused, embedded in paraffin, and sliced for histology. Brain slices from the hippocampal area and fimbria of the hippocampus were stained for myelinated axons with Luxol fast blue and counterstained with eosin, and stained with anti-CD3 antibodies for the presence of autoimmune effector T cells and counterstained with hematoxylin. (4a) Brain slices from maladapted mice showed no staining for effector T cells (ii and iv). (4b) Brain slices from the well-adapted mice showed autoimmune T-cell reactivity in hippocampal areas (ii and iv) corresponding to myelin reactivity (Luxol-positive areas; i and iii). (4c) WT mice that were not exposed to stress showed, as expected, no T-cell reactivity in brain slices. Micrographs show representative results of at least six brain slices from each mouse, and from at least three mice in each group.

**Figs. 5a-5c** show that dopamine (DA) reduces the suppressive activity mediated by Treg. Proliferation of Teff was assayed by incorporation of [ $^3H$ ]-thymidine into Teff co-cultured with naturally occurring Treg. Recorded values are from one of three representative experiments and are expressed as means  $\pm$  SD of four replicates. (5a) Treg were activated by incubation for 24 hr with anti-CD3 antibodies in the presence of mouse recombinant interleukin (mrIL)-2. Incubation of the activated Treg for 2 hr with DA ( $10^{-5}M$  or  $10^{-7}M$ ) before their co-culturing with Teff reduced their suppression of Teff compared with that obtained with Treg not

exposed to DA. (5b) DA ( $10^{-5}$ M,  $10^{-7}$ M, or  $10^{-9}$ M) added to freshly purified Treg. DA ( $10^{-5}$ M,  $10^{-7}$ M) had a similar effect on activity of naïve Treg to that of activated Treg, whereas the effect of dopamine at  $10^{-9}$ M on Treg-mediated suppression was not significant. (5c) Activation of Treg for 96 hr, followed by  
5 addition of DA ( $10^{-5}$ M) for 2 hr at the end of activation, significantly reduced the suppressive activity of Treg on Teff. Incubation of Teff with DA ( $10^{-5}$  M) for 2 hr did not affect their susceptibility to Treg-induced suppression.

**Figs. 6a-6d** show that the dopamine (DA) effect on Treg is mediated via D1-type receptor family. Proliferation of Teff was assayed by incorporation of [ $^3$ H]-  
10 thymidine into Teff cocultured with naturally occurring Treg. Recorded values are from one of three representative experiments and are expressed as means  $\pm$  SD of four replicates. (6a) Addition of norepinephrine (NE;  $10^{-5}$  or  $10^{-7}$ M) to Treg for 2 hr after their activation for 24 hr did not affect the suppressive activity of Treg. Significant differences between groups were analyzed by Student's *t* test ( $P < 0.001$ ).  
15 (6b) The inhibitory effect of DA on the suppressive activity of Treg was mimicked by SKF-38393, a specific agonist of the D1-type family. The D2-type agonist quinpirole did not alter the effect of dopamine on Treg. SCH 23390, a specific D1-type antagonist, wiped out the dopamine effect on the suppressive activity of Treg. Each experiment was performed at least five times, and representative results are  
20 shown. (6c) Incubation of Treg or Teff with dopamine did not cause apoptosis, as shown by propidium iodide staining for DNA content and FACS analysis of Treg and Teff, 48 hr after their incubation for 2 hr with dopamine. (6d) Staining for apoptosis with annexin V for phosphatidylserine on a surface membrane. No increase in annexin V-labeled cells was detected on incubation of Treg with  
25 dopamine or with the D1-type agonist SKF-38393.

**Figs. 7a-7f** show a preferential expression of D1-type receptors by Treg. Expression of dopamine receptors on Treg and Teff was investigated on mRNA and protein levels. (7a,7b) Semiquantitative reverse transcription-PCR (RT-PCR) analysis for D1-R and D5-R expression. mRNA was extracted from freshly isolated  
30 Teff and Treg. The housekeeping gene  $\beta$ -actin was used for quantitative analysis.

The results of one of five representative experiments are shown. (7c) Quantitative real-time PCR using primers for D1-R and D5-R to verify the differences in the expression of dopamine receptors on Teff and Treg. The results presented are arbitrary units and are from one of three representative experiments performed. (7d, 5 7e) Semiquantitative RT-PCR for D2-R, D3-R, and D4-R expression. mRNA was extracted from freshly purified Teff and Treg. The housekeeping gene  $\beta$ -actin was used for quantitative analysis. The results of one of five representative experiments are shown. (7f) Representative micrographs of D1-R-immunoreactive T-cells using fluorescence and confocal microscopy. Also shown are micrographs stained with 10 Hoechst and visualized by fluorescence microscopy. D1-R immunoreactivity was observed in Treg but not in Teff.

**Figs. 8a-8d** show the molecular mechanism underlying the effect of dopamine on Treg. (8a) Expression of CTLA-4. Treg were activated for 24 hr, then incubated for 2 hr with dopamine or SKF-38393 (control cells were activated but 15 were not incubated with either dopamine or SKF-38393; note that different cell preparations were used for each treatment, and therefore the controls used for each treatment were not the same) and were stained 24 hr later for CTLA-4 on cell surfaces. CTLA-4 expression was reduced after exposure to dopamine or to SKF-38393. Representative results of one of five independent experiments with each 20 treatment are shown. (8b) Production of IL-10. Treg were activated for 24 hr with anti-CD3 and IL-2 in the presence of lethally irradiated splenocytes (APCs) and then for an additional 2 hr with dopamine. Conditioned media were collected after 24, 48, or 72 hr of culture and were assayed for IL-10 using a sandwich ELISA. At any given time, significantly less IL-10 was detected in media conditioned by 25 dopamine-treated Treg than in media conditioned by Treg not exposed to dopamine. Statistical significance was verified using Student's *t* test analysis (\*\* $P < 0.01$ ; \* $P < 0.05$ ). The results shown are of one of three independent experiments, performed at each time point. (8c) Lack of IL-2 production by Treg. Treg and Teff were activated separately for 48 hr with anti-CD3 and anti-CD28 (without mIL-2) 30 with or without dopamine. Conditioned media were collected after 48 hr and

subjected to ELISA. Treg with or without dopamine did not secrete detectable levels of IL-2. Production of IL-2 by Teff was not affected by dopamine. (8d) Foxp3 expression in Treg. Treg were activated for 24 hr with anti-CD3 and anti-CD28 in the presence of IL-2, then exposed to dopamine for 2 hr, washed, and  
5 analyzed 30 min later for Foxp3 expression. No changes in Foxp3 were detected after 30 min of dopamine treatment of naive Treg.

**Figs. 9a-9b** show that ERK1/2 phosphorylation inhibitors downregulate Treg-suppressive activity. (9a) Treg were activated by incubation for 30 min with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 and in the presence or  
10 absence of a tyrosine kinase inhibitor (genistein) and were then cocultured with Teff. The suppression of Teff by Treg was significantly reduced in the presence of genistein. (9b) Similarly, incubation of activated Treg with the specific MEK inhibitor PD98059, which inhibits the ERK1/2 signaling pathway, almost completely abolished their suppression of Treg.

**Figs. 10a-10c** show the correlation between Treg activity and activation state of ERK1/2. (10a, 10b) Western blot analyses of Treg lysates after activation for 20 min with anti-CD3 and anti-CD28, in the presence or absence of dopamine (10a) or SKF-38393 (10b). After activation, the amounts of phospho-ERK1/2 seen in Treg are larger than in Teff (Fig. 10a) but are reduced by dopamine (10a) or by SKF-  
15 38393 (10b). Dopamine did not cause a significant change in phospho-ERK1/2 levels in Teff (10a, 10b). (10c) Quantitative analysis of phospho-bands using NIH Image version 1.62.

**Figs. 11a-11d** show that dopamine alters the adhesive properties of Treg. (11a) Treg and Teff were activated for 24 hr with anti-CD3 and anti-CD28 and were  
25 then incubated, with or without dopamine ( $10^{-5}$  to  $10^{-9}$  M), for 2 hr. In the absence of dopamine, adhesion of Treg to the chondroitin sulfate proteoglycans (CSPG) matrix was significantly stronger than that of Teff. Incubation with dopamine significantly reduced the adhesion of Treg in a concentration-dependent manner. The effect of dopamine on Treg adhesion could be mimicked by SKF-38393. The  
30 dopamine effect was blocked by SCH-23390, a D1-type antagonist. Dopamine did

not significantly alter the adhesion of Teff. A Mann–Whitney nonparametric *U* test was used for statistical analysis. (11b) In the absence of dopamine, adhesion of Treg to fibronectin was only slightly (but still significantly) stronger than that of Teff. However, dopamine did not significantly alter the adhesion of either Treg or Teff. A Mann–Whitney nonparametric *U* test was used for statistical analysis. (11c) Treg were activated for 30 min in the presence or absence of the ERK1/2 signaling pathway inhibitor PD98059 and then subjected to an adhesion assay to CSPG. Adhesion of Treg incubated with PD98059 was significantly weaker than that of control Treg cells. (11d) CD44 expression in Treg and in Teff. FACS analysis showed that significantly larger amounts of CD44 are expressed in Treg than in Teff. After incubation with dopamine, CD44 expression was significantly decreased in Treg but was not affected in Teff.

**Figs. 12a-12e** show that dopamine alters the migratory properties of Treg. (12a) The total population of purified CD4<sup>+</sup>T-cells was subjected to a migration assay. The percentage of CD4<sup>+</sup>CD25<sup>+</sup>T-cells in the total population after migration toward CCL22 (macrophage-derived chemokine; MDC) was significantly higher than in the original population. Exposure of Treg to dopamine significantly decreased their migration toward MDC. Migration of Teff toward stromal cell-derived factor-1 (SDF-1) was significantly stronger than that of Treg, and neither Teff nor Treg migration was affected by exposure to dopamine. A Mann–Whitney nonparametric *U* test was used for statistical analysis. (12b, 12c) Migration of purified Treg was significantly decreased after incubation of Treg with dopamine. Treg in the lower (postmigration) chamber were collected and counted by FACS for a defined time period after staining for membrane CD4 marker. Values are representative results of the FACS analysis (12b), and the mean number of cells from triplicates of the same experiment are shown in 12c,d,e. Semiquantitative reverse transcription-PCR for CCR-4 expression in Treg and Teff. mRNA was isolated from Teff and Treg, incubated for 2 hr with or without dopamine. The PCR products were quantified (12e) relative to a housekeeping gene ( $\beta$ -actin). The

results of one representative experiment are shown (12d). Each experiment was performed in triplicate and repeated at least three times. \*\*\* $P<0.01$ ; \*\* $P<0.01$ .

**Figs. 13A-13C** show that systemic injection of dopamine, through downregulation of Treg activity, increases neuronal survival after optic nerve crush injury. (13a) BALB/c mice were given injections of dopamine (0.4 mg/kg) immediately after being subjected to a partial crush injury of the optic nerve. Two weeks later, their retinas were excised and the numbers of surviving neurons were determined (see Materials and Methods). Significantly more neurons survived in dopamine-injected mice than in vehicle-injected controls ( $P<0.01$ ; Student's  $t$  test).

Bars represent mean numbers of retinal ganglion cells per square meter of the retina. Each experiment was performed twice;  $n=6-8$  mice in each group. A two-tailed Student's  $t$  test was used for statistical analysis; \*\*\* $P>0.001$ ; \*\* $P>0.01$ .

(13b) Neuronal survival was significantly worse in BALB/c mice that were inoculated (immediately after their exposure to a toxic excess of intraocular glutamate) with activated Treg than in Teff-inoculated mice. Neuronal loss is expressed as a percentage of the number of neurons in untreated glutamate-injected controls. Neuronal survival in BALB/c mice that were exposed to a toxic excess of intraocular glutamate and then treated with activated Treg that were incubated for 2hr with  $10^{-5}$  M dopamine before being administered *in vivo* did not differ from that in vehicle-treated glutamate-injected mice. (13c) Representative micrographs of retinas from mice given injections of glutamate and either Teff or Treg. Each experiment was performed twice;  $n=6-8$  mice in each group. A two-tailed Student's  $t$  test was used for statistical analysis; \*\*\* $P<0.001$ .

**Fig. 14** is a graph showing that administration of dopamine with the D2-R antagonist clozapine increases neuronal survival after glutamate-induced neuronal cell death.

**Figs. 15A-15C** show that low-dose total-body  $\gamma$ -irradiation (TBI) increases neuronal survival after CNS mechanical injury. Young adult female rats of the Sprague-Dawley (SPD) and Lewis strains were subjected to low-dose TBI immediately after unilateral optic nerve crush injury. Two weeks later a fluorescent

dye was applied, and after 5 more days retinas were excised and whole-mounted (see Materials and Methods). Irradiation induced significant neuroprotection in both SPD (15A) and Lewis (15B) strains ( $P < 0.01$ ; Student's  $t$ -test). (15C) TBI of rats devoid of T cells due to neonatal thymectomy had no effect on the extent of secondary degeneration. Results are presented as the increase in neuronal survival in the treated animals, expressed as a percentage of that in untreated controls. Mean values for each group (error bars indicate SEM) are given.

**Fig. 16** shows that low-dose TBI improves spontaneous recovery from spinal cord injury. SPD rats were deeply anesthetized and subjected to severe spinal cord contusion at T8 (NYU impactor, 10 g, 50 mm). One group was subjected to a single low dose of TBI 2 days after the contusion, and the other group was left untreated. Recovery was assessed by the BBB open-field locomotor rating scale at the indicated times. Results are expressed as mean values for each group (error bars indicate SEM). Statistical differences were calculated by two-tailed  $t$ -test;  $*P < 0.05$ ,  $***P < 0.001$ ).

**Figs. 17A-17G** show that low-dose TBI induces lymphopenia and activates T cells. (17A) Isolated Treg and Teff were each cultured for 48 h, then subjected to low-dose TBI (350 rad), which was followed by a further 48 h in culture. The T cells were then stained with propidium iodide (PI) and analyzed for dead cells by FACSsort. The numbers of dead cells did not differ significantly in Teff and Treg control populations. After  $\gamma$ -irradiation, however, significantly more Treg died ( $68.4 \pm 5.6\%$ ) than Teff ( $23.3 \pm 2.5\%$ ). (17B) Photograph of spleens excised from naïve and irradiated rats 1, 3, and 7 days after TBI (bar = 8 mm). The spleen tissue showed a significant decrease in size 3 days after  $\gamma$ -irradiation and showed signs of recovery from day 7 after TBI. (17C) Three days after TBI, lymphocytes were prepared from the axial, mesenteric, and salivary lymph nodes, the spleens, and the peripheral blood of  $\gamma$ -irradiated and control rats. The cells were stained for the  $\alpha$ -chain of IL-2R (CD25), an activation marker (ergotype). (17D) A significant increase in CD25 mRNA was observed 3 days and 7 days after TBI. Analysis of mRNA obtained from  $\gamma$ -irradiated rats revealed up-regulated expression of mRNAs

for the proinflammatory cytokines (17F) IFN- $\gamma$  and (17G) IL-12 after TBI. IFN- $\gamma$  was significantly increased 3 day after  $\gamma$ -irradiation, whereas IL-12 was up-regulated on day 7 after induction of lymphopenia.

**Figs. 18A-18C** show that low-dose total-body or lymphoid organ  $\gamma$ -irradiation increases resistance to glutamate-induced neuronal toxicity: Involvement of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. (18A) C57Bl/6J mice were subjected to TBI and BALB/c mice to TLI immediately after intravitreal injection of a toxic dose of glutamate. Neuronal survival was measured 1 week later by application of a fluorescent dye and subsequent counting of labeled retinal ganglion cells (RGCs) in whole-mounted retinas. A single dose of TBI, applied immediately after or 6 or 14 days before administration of a toxic dose of glutamate, significantly increased the resistance of RGCs to glutamate-induced neurotoxicity ( $P < 0.001$ ,  $P < 0.01$  and  $P < 0.001$ , respectively; Student's *t*-test).  $\gamma$ -irradiation of SCID mice had no effect on neuronal survival. (18B) After TLI, significantly more neurons survived in the irradiated than in the nonirradiated mice ( $P < 0.001$ ). Results are presented as the increase in neuronal survival in the treated animals, expressed as a percentage of that in untreated controls. (18C) After retinal exposure to glutamate neurotoxicity followed immediately by TBI (300 rad), mice in one group were injected with Treg and mice in another group with Teff. The bar graph shows the percentage increase in neuronal survival after TBI and treatments compared to those in nonirradiated controls. Injection of Treg wiped out this effect. Injection of Teff did not interfere with the beneficial effect.

**Figs. 19A-19B** show that incubation of activated Treg for 2 h with poly-YE prior to their co-culturing with Teff (TregYE) alleviated the Treg suppressive activity on Teff, as measured by the resulting proliferation of Teff, compared to that obtained with activated Treg not exposed to poly-YE (control). **Fig. 16B** shows that the effect was even more significant in the co-cultures of Teff and TregYE to which poly-YE was added (TregYE+YE), as shown by the significantly higher Teff proliferation.

**Figs. 20A-20D** show that the cytokine phenotype of the Treg cells is changed in the presence of poly-YE and it becomes similar to the phenotype of Teff cells: there is up-regulation of IFN- $\gamma$  (20A), TGF- $\beta$  20(B) and IL-2 (20C) and down-regulation of IL-10 (20D).

5        **Fig. 21** shows tracking of poly-YE-injected (left panels) and PBS-injected control mice (right panels) in the Morris water maze (MWM) after injection of the psychotomimetic drug MK-801.

10        **Fig. 22** shows performance of a spatial memory task in the MWM after injection of the psychotomimetic drug MK-801, of poly-YE-injected (diamonds) and PBS-injected control mice (squares).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for treatment of an individual suffering from a psychiatric disorder which comprises administering to said individual in need of such a treatment an effective amount of an agent that causes down-regulation of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells on CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (Teff), modulation of the immune response and/or modulation of autoimmune response, but excluding Copolymer 1, a Copolymer 1-related peptide and Copolymer 1-related polypeptide, and further excluding a combination of dopamine, a dopamine precursor or an agonist of the dopamine receptor type 1 family (D1-R agonist) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist), when said individual is one suffering from schizophrenia.

25        In one embodiment, the invention relates to a method for down-regulation of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) on CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (Teff) in an individual suffering from a psychiatric disorder, which comprises administering to said individual in need an amount of an agent that causes said down-regulation of Treg, but excluding Copolymer 1, a Copolymer 1-related peptide and Copolymer 1-related polypeptide, and further excluding a combination of dopamine, a dopamine precursor or an agonist of the dopamine

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receptor type 1 family (D1-R agonist) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist), when said individual is one suffering from schizophrenia.

In one embodiment of the invention, the agent that causes down-regulation of the Treg cells is low dose whole-body or total lymphoid organ  $\gamma$ -irradiation. Preferred doses will be in the range of 200-350 Rad and the patient may be irradiated several times at intervals of time as determined by the physician, according to the patient's condition.

In another embodiment of the invention, the agent that causes down-regulation of the Treg cells is selected from the group consisting of (i) dopamine; (ii) a dopamine precursor; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist); (iv) a combination of (i) and (ii); and (v) a combination of (i), (ii) or (iii) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist), provided that when said agent is a combination of (i), (ii) or (iii) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist), said individual is not one suffering from schizophrenia.

As used herein, the terms "dopamine", "dopamine precursor", "D1-R agonist" and "D2-R antagonist" are meant to include the compounds themselves as well as their pharmaceutically acceptable salts.

In one most preferred embodiment, the agent is dopamine that can be administered parenterally by injection.

According to the present invention, dopamine will not replace the dopamine in the brain but will down-regulate the suppressive effect of Treg cells on Teff cells in the periphery, thus allowing the Teff cells to exhibit the protective autoimmunity that is necessary to cope with the stressful condition. Thus, according to the invention, dopamine can be used in combination with its precursor levodopa, optionally in further combination with carbidopa, even for the treatment of a Parkinson's patient in need of a treatment for a psychiatric disorder.

In another embodiment, the agent is a dopamine D1-R agonist. In a further embodiment, the agent is a combination of dopamine with a dopamine D2-R

antagonist. In still a further embodiment, the agent is a combination of dopamine D1-R agonist with a dopamine D2-R antagonist.

The dopamine D1-R agonist may be any such agonist known or to be developed in the future and includes, without being limited to, a D1-R agonist selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine. Preferably, the D1-R agonist is SKF-38393 and its hydrochloride salt [(+/-)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol.HCl].

The dopamine D2-R antagonist may be any such antagonist known or to be developed in the future and includes, without being limited to, a D2-R antagonist selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2. Preferably, the D2-R antagonist is clozapine.

In a further preferred embodiment, the agent is a combination of dopamine with a dopamine D2-R antagonist, preferably dopamine and clozapine.

According to a further embodiment of the invention, the agent is one that causes modulation of the autoimmune response such as, but not limited to, a modified CNS-peptide obtained by modification of a self-peptide derived from a CNS-specific antigen, which modification consists in the replacement of one or more amino acid residues of the self-peptide by different amino acid residues, said modified CNS peptide still being capable of recognizing the T-cell receptor recognized by the self-peptide but with less affinity.

The modified peptide may be derived by modification of a self-peptide derived from a CNS-specific antigen selected from the group consisting of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors. The modified CNS peptide may be obtained by modification of an immunogenic epitope or a cryptic

epitope of said CNS-specific antigen. The modified peptide has from 9 to 20, preferably 9-18, 9-15 or 9-13, amino acid residues.

In one preferred embodiment, the CNS-specific antigen is MBP and the modified peptide is obtained by modification of a peptide selected from the group consisting of p11, p51-70, p87-99, p91-110, p131-150, and p151-170 of MBP.

In preferred embodiments, the modified MBP peptide is obtained by modification of the self-peptide p87-99 of MBP VHFFKNIVTPRTP [SEQ ID NO:1] by replacing the lysine residue 91 with glycine (G91), to give a peptide of the sequence: VHFFGNIVTPRTP [SEQ ID NO:2], or with alanine (A91):  
 10 VHFFANIVTPRTP [SEQ ID NO:3], or by replacing the proline residue 96 with alanine (A96): VHFFKNIVTARTP [SEQ ID NO:4].

In another preferred embodiment, the CNS-specific antigen is a mammalian Nogo or Nogo receptor molecule. In one embodiment, the modified peptide is obtained by modification of the peptide designated p472 containing the residues  
 15 623-640 of rat Nogo-A of the sequence S Y D S I K L E P E N P P P Y E E A [SEQ ID NO:5], in which the Lys 628 residue is replaced by Val or Ala or another similar residue, preferably the peptides:

S Y D S I V L E P E N P P P Y E E A [SEQ ID NO:6]

S Y D S I A L E P E N P P P Y E E A [SEQ ID NO:7]

20 In another embodiment, the modified peptide is obtained by modification of the peptides derived from the Nogo receptor of the sequences: S G V P S N L P Q R L A G R D [SEQ ID NO:8] or T R S H C R L G Q A G S G S S [SEQ ID NO:9] in which an Arg residue is replaced by Val or Ala or another similar residue, preferably the peptides:

25 S G V P S N L P Q V L A G R D [SEQ ID NO:10]

S G V P S N L P Q R L A G V D [SEQ ID NO:11]

S G V P S N L P Q A L A G R D [SEQ ID NO:12]

S G V P S N L P Q R L A G A D [SEQ ID NO:13]

T V S H C R L G Q A G S G S S [SEQ ID NO:14]

30 T R S H C V L G Q A G S G S S [SEQ ID NO:15]

T A S H C R L G Q A G S G S S [SEQ ID NO:16]

T R S H C A L G Q A G S G S S [SEQ ID NO:17]

In another preferred embodiment of the invention, the agent that causes modulation of the autoimmune response are T cells that have been activated either  
5 by a CNS-specific antigen or by a modified peptide derived from a CNS-antigen as defined above. T cells for use in the invention that have been activated by said CNS-antigens are described in WO 99/60021, WO 02/055010 and WO 03/002602.

In yet a further preferred embodiment of the invention, the agent is the copolymer poly-YE or a poly-YE related peptide or polypeptide that causes down-  
10 regulation of the suppressive activity of the cells, modulation of the immune response and/or modulation of autoimmunity response. Poly-YE, as defined herein, is a random copolymer of Tyr and Glu that may contain the amino acids Glu and Tyr in any available ratio such as, for example, poly-(Glu, Tyr) 1:1 and poly(Glu, Tyr) 4:1. The modulation of the immune response and modulation of autoimmunity  
15 response by poly-YE is described in US Application No. 10/807414 and in WO 03/002140. As used herein, the term "poly-YE related peptide or polypeptide" refers to random copolymers of Tyr and Glu with different ratios of Glu and Tyr and/or different molecular weight and to random peptides containing several residues of Tyr and Glu.

20 Examples of psychiatric or mental disorders that can be treated according to the invention include, but are not limited to: (i) anxiety disorders, that include phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic  
25 disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder, schizophreniform disorder, schizoaffective disorder and delusional disorder; (iv) drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, and phencyclidine use; and and (v) memory loss disorders such as amnesia or memory  
30 loss associated with Alzheimer's type dementia or with non-Alzheimer's type

dementia, e.g. multi-infarct dementia or memory loss associated with Parkinson's disease, Huntington's disease, Creutzfeld-Jakob disease, head trauma, HIV infection, hypo-thyroidism and vitamin B12 deficiency.

5 In preferred embodiments, the psychiatric disorder is schizophrenia, an anxiety disorder such as stress or post-traumatic stress disorder, or a mood disorder such as depression or a bipolar disorder.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be  
10 "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered.

When the pharmaceutical composition comprises a combination of agents, for example dopamine and levodopa, optionally with carbidopa, each of the agents  
15 may be presented in a separate container, and the resulting article of manufacture will contain a leaflet with instructions to the physician and to the patient about the order and dosage of administration of the agents.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral,  
20 intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

As will be evident to those skilled in the art, the therapeutic effect depends at times on the condition or disease to be treated, on the individual's age and health condition, on other physical parameters (e.g., gender, weight, etc.) of the individual,  
25 as well as on various other factors, e.g., whether the individual is taking other drugs, etc., and thus suitable doses and protocols of administration will be decided by the physician taking all these factors into consideration.

All patents and patent applications cited in the specification are herewith incorporated by reference as if fully disclosed herein.

The invention will now be illustrated by the following non-limiting examples and accompanying figures.

### EXAMPLES

5       The animals used in the experiments, if not indicated differently, were supplied by the Animal Breeding Center of the Weizmann Institute of Science (Rehovot, Israel). All animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee (IACUC).

10

### SECTION I

**Protection against consequences of psychological trauma is T-cell dependent and is suppressed by naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg)**

15       Protection against neurodegenerative conditions in the CNS is T-cell dependent. Here we examined whether T cells also play a role in the ability of mice to withstand psychological stress (caused, for example, by predator odor) associated with behavioral changes reminiscent of post-traumatic stress disorder (PTSD). Measurement of behavioral adaptation (acoustic startle response and avoidance behavior) in mice after their exposure to predator odor revealed that maladaptation  
20       was significantly more prevalent ( $\chi^2=10.6$ ,  $P<0.001$ ) in immune-deficient mice (62%) than in their wild-type counterparts (17%). The prevalence of maladaptation in the normal mice was reduced upon removal of naturally occurring Treg cells, which normally suppress autoimmunity. The ability to cope with stress was correlated with recruitment of T cells in the brain. These findings suggest that a  
25       well-controlled T cell-dependent dialog between the brain and the immune system is needed for *mens sana in corpore sano*.

### Materials and Methods (Section I)

30       *(i) Animals.* Inbred adult wild-type (WT), SCID and *nu/nu* BALB/c, C57Bl/6J mice and TgT<sub>MBP</sub> and TgT<sub>MBP</sub>/RAG1<sup>-/-</sup> were housed in cages, 10 mice per

cage, in an animal room at a stable temperature and with a reversed 12-hour light/dark cycle. Food and water were supplied ad libitum. All tests were carried out in dim light during the dark phase.

(ii) *Experimental stress paradigm.* Mice to be tested (experimental group) were placed for 10 minutes on thoroughly soiled cat litter (used by a cat for 2 days and sifted for feces). WT control mice were exposed for 10 minutes to unused litter.

***Behavioral testing:***

(iii) *Elevated plus-maze (EPM).* The maze we used was a black opaque perspex platform with four arms in the shape of a plus, elevated 78 cm above the ground, as described by File (Griebel et al., 1995). Each arm was 24 cm long and 7.5 cm wide. One pair of opposite arms was “closed”, i.e., the arms were enclosed by 20.5-cm-high perspex walls on both sides and on the outer edges of the platform, and the other pair was “open”, surrounded only by a 3-mm-high perspex lip, which served as a tactile guide for animals in the open areas. The apparatus was illuminated by dim red lighting that provided 40–60 lux in both the open and the closed arms. Mice were placed one at a time in the central platform, facing towards different arms on different days in randomized order. Between each test session the maze was cleaned with an aqueous solution of 5% ethanol and dried thoroughly.

Behavior on the EPM was recorded using EthoVision programs (Noldus) that recorded the location of the mouse over the 5-minute test period. To ensure that the software provided accurate monitoring of the various parameters selected for analysis, videotaped replay of the behavior of randomly chosen mice was scrutinized by an experienced observer.

Five behavioral parameters were assessed: (1) time spent in the open arms; (2) time spent in the closed arms; (3) number of entries into open arms; (4) number of entries into closed arms; (5) total number of entries into all arms. Mice were recorded as having entered an open or closed arm only when all four paws had passed over the dividing line between open and closed arms. The number of entries into any arm of the maze (total arm entries) was defined as ‘exploration activity’.

(iv) *Acoustic startle response (ASR)*. Pairs of mice were tested in startle chambers. The ASR and pre-pulse inhibition were measured using two ventilated startle chambers (SR-LAB system, San Diego Instruments, San Diego, CA). Each chamber consists of a Plexiglas cylinder resting on a platform inside a ventilated sound-attenuated chamber. A high-frequency loudspeaker inside the chamber produces both a continuous broad-band background noise of 68 dB and different acoustic stimuli. Movement inside the tube is detected by a piezoelectric accelerometer located below the frame. The amplitude of the ASR of the whole body to an acoustic pulse was defined as the average of 100 accelerometer readings, 100 ms each, collected from pulse onset. These readings (signals) were digitized and stored in a computer. Sound levels within each test chamber are routinely measured using a sound-level meter (Radio Shack, San Diego Instruments) to ensure consistent presentation. An SR-LAB calibration unit was used routinely to ensure consistency of the stabilimeter sensitivity between test chambers and over time (Swerdlow and Geyer, 1998). The mice were placed inside the tube, and the startle session started with a 5-minute period of acclimatization to the background noise level of 68 dB, which was maintained throughout the session.

(v) *Study design for determination of “cut-off behavioral criteria” (CBC)*.

The studies were designed in two steps:

Step I – Prior to attempting to distinguish the differentially affected subgroups, we routinely perform a preliminary assessment of the overall response of the exposed population intended to ascertain the accuracy of our zero-hypothesis, i.e. to demonstrate that exposure to the stressor did in fact have significant overall behavioral effects on the exposed animals as a group compared to controls, in each of our studies. The data are also ascertained to demonstrate a range of varying degrees of behavioral changes.

Behavioral changes, such as extremely compromised exploratory behavior on the plus maze and markedly increased startle reaction that does not undergo any adaptation reflect anxiety-like behaviors, i.e. fearfulness and hypervigilance. In keeping with the work of Blanchard and Blanchard (Blanchard et al., 1990;

Blanchard et al., 1993; Blanchard et al., 1998), Adamec (Adamec et al., 1998; Adamec et al., 1999a) and Cohen (Cohen et al., 1996; Cohen et al., 2000; Cohen et al., 2003) the observed behaviors at this time-point are considered to reflect relatively long-term and persistent changes. Since it has as yet not been possible to  
5 design an animal model for the intrusive cluster of symptoms, changes such as these, which persist over the space of a week or more, are considered to represent a fair representation of PTSD-like symptoms in terms of animal models.

Step II - The CBC applied to exposed animals:

Having established that the stressor had an effect on the animals and that not  
10 all animals responded to it in the same manner, we focus only on animals that demonstrate extremes of behavioral change on the one hand or virtually no change on the other.

In order to maximize the clarity of which animals to define as “affected”, and so to minimize the chance of including “false positives”, we define the behavioral  
15 cut-off criteria to represent the most extreme degree of behavioral disturbance in each of two consecutive behavioral paradigms. In order to be defined as “affected”, the individual animal has to have conformed to both sets of criteria, consecutively. Conversely, in order to be considered to have responded hardly at all, animals must conform to equally extreme criteria for “near normal” behaviors. The validity of the  
20 criteria are re-affirmed in each study by ascertaining that the vast majority of unexposed control animals conform to the latter and none, or almost none, to the former. The CBCs determined as above, were as follows: **(a) Maladapted:** 1) Five minutes spent in closed arms and zero (0) entries into the open-arms; 2) Mean amplitude of the startle response (at 110 Db) > 800 units and nonhabituation of the  
25 acoustic startle response; **(b) Well-Adapted:** 1) 0-1 minutes spent in closed arms and  $\geq 8$  open-arm entries; 2) Mean amplitude of the startle response (at 110 Db) < 600 units and normal habituation of the acoustic startle response.

*(vi) Supplemental Information.* The behavioral outcome in mice exposed to the odor of a predator has been used as a model for PTSD (Adamec et al., 1999b;  
30 Cohen et al., 2003). Brief, escapable exposure of mice or rats to a cat or cat odor

increases the defensive behaviors observed in a visible burrow system for many hours after removal of the threat (Rodgers et al., 1990). The long-lasting behavior abnormality is being viewed as maladaptation to the predator stress (i.e. PTSD). The stressor and the time scale used in the present study might justify view the results as relevance to PTSD according to the following criteria (Yehuda and Antelman, 1993): (a) The stressor is strong and transient and provides a more natural setting than that offered by other types of stressors, such as electrical shocks to the tail (Adamec et al., 1997). (b) The observed reduction in time spent by the stressed mice in the open arms of the EPM is reminiscent of the avoidance behavior seen in patients with PTSD. Stressed mice did not show fewer total entries in the EPM than unstressed mice. This finding is consistent with anxiety rather than with nonspecific impairment of locomotion. The DSM-IV defines this symptom as the persistent avoidance of reminders of the trauma and the numbing of responsiveness. Since the traumatic event in these mice took place in an open space, it is in line with this definition. (c) Seven days in the life of a mouse that normally lives for 3 years is roughly equivalent to 6 months in a human life span of 72 years (Adamec et al., 1997). It thus seems that our assessment of mice 7 days after the trauma indeed points to PTSD rather than to an acute stress reaction.

**(vii) Antibodies and reagents.** Mouse recombinant IL-2 (mrIL-2) and anti mouse  $\zeta$ -CD3 (clone 145-2C11) were purchased from R&D Systems (Minneapolis, MN). Rat anti-mouse phycoerythrin (PE)-conjugated CD25 antibody (PC61) was purchased from Pharmingen (Becton-Dickinson, Franklin Lakes, NJ).

**(viii) Preparation of splenocytes.** Donor splenocytes from rats (aged up to 10 weeks) were obtained by rupturing the spleen and following conventional procedures. The splenocytes were washed with hypotonic buffer (ACK) to lyse red blood cells.

**(ix) Preparation of lymphocytes.** Mouse donor lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) were ruptured through mesh. The lymphocytes were washed with ACK buffer to lyse red blood cells.

**(x) Purification of murine  $CD4^+CD25^+/CD4^+CD25^-$  T cells.** Lymph nodes

(axillary, inguinal, superficial cervical, mandibular, and mesenteric) and spleens were harvested and mashed. T cells were purified (enriched by negative selection) on T-cell columns (R&D Systems). The enriched T cells were incubated with anti-CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and negatively selected CD4<sup>+</sup> T cells were incubated with PE-conjugated anti-CD25 (30 µg/10<sup>8</sup> cells) in PBS/2% fetal calf serum. They were then washed and incubated with anti-PE microbeads (Miltenyi Biotec) and subjected to magnetic separation with AutoMACS (Miltenyi Biotec). The retained cells were eluted from the column as purified CD4<sup>+</sup>CD25<sup>+</sup> cells. The negative fraction consisted of CD4<sup>+</sup>CD25<sup>-</sup> T cells. Cell purity was checked by FACSsort (Becton-Dickinson) and typically ranged from 88% to 95%. Purified cells were cultured in 24-well plates (1 ml) with T cell-depleted spleen cells as accessory cells (irradiated with 3000 rad) and 0.5 µg/ml anti-CD3, supplemented with 100 units of mouse recombinant IL-2 (R&D Systems).

**(xi) Histology and immunohistochemistry of paraffin-embedded brain sections.** Paraffin-embedded brain tissues from maladapted nude mice replenished with a normal population of wild-type splenocytes, or from well-adapted nude mice replenished with wild type splenocytes depleted of CD25<sup>+</sup> regulatory T cells, were cut into 4-µm-thick coronal sections, deparaffinized with xylene, and dehydrated with a graded series of ethanol solutions. The sections were then stained with Luxol fast blue (Sigma-Aldrich, Israel) and counterstained with Fast Red (Sigma-Aldrich, Israel). For immunohistochemical analyses, deparaffinized and dehydrated sections were immersed for 30 min in methanol containing 3% H<sub>2</sub>O<sub>2</sub> and 1% concentrated HCl to block endogenous peroxidase activity, then treated for 1 hour with phosphate-buffered saline (PBS), pH 7.4, containing 20% normal rabbit serum and 0.3% Triton X-100, and incubated overnight at room temperature with anti-CD3 antibodies (Serotec, Oxford, UK; diluted 1:50 in PBS containing 2% normal rabbit serum). The sections were washed with PBS and incubated for 30 min, first with biotinylated anti-rabbit IgG and then with avidin-biotinylated peroxidase complex

(Vector Laboratories, Burlingame, CA). Peroxidase activity in a solution of 3,3'-diaminobenzidine was visualized by light microscopy.

**Example 1. Adaptation to acute psychological stress is T-cell dependent**

5        Exposure of rats or mice to a predator (cat) or odor of a predator (thoroughly soiled cat litter) for 10 minutes causes major stress in these animals (Adamec et al., 1999b; Cohen et al., 2000). To test an assumption that adaptation to psychological stress is dependent on T cells, we used mice depleted of mature T cells and compared them to WT mice with properly functioning immune systems. We used  
10    BALB/c strain to compare the stress response of the WT to that of mice with the same genetic background but suffering from SCID. Significantly more SCID mice than WT mice showed symptoms of maladaptation (61.9% compared to 17.2%;  $\chi^2 = 10.6$ ,  $P < 0.001$ ; **Fig. 1a**). The same comparison between nude mice (depleted of mature T cells only) and the WT yielded similar results (70% compared to 17.2%;  
15     $\chi^2 = 13.9$ ,  $P < 0.0002$ ; **Fig. 1a**), verifying that the observed differences were due to the absence of mature T cells. Differences between WT mice and SCID or nude mice were significantly manifested in the ASR (**Fig. 1b**) as well as in the time spent in the closed arms of the EPM (**Fig. 1c**).

20    **Example 2. T cell required to withstand mental trauma are autoimmune T cells reactive to CNS-specific myelin-associated proteins**

      The above results suggested that perhaps T cells are needed for the functional maintenance of higher brain functions; such dependencies can hardly be demonstrated in resting stage but are evident under stress conditions. We  
25    hypothesized that if this is the case, cells that can home to site of stress should be the cells that contributed to the observed effect. Since homing and functioning of T cells means recognition and therefore specificity, we made an assumption that perhaps the relevant T cells are T cells that recognize abundant and highly distributed self-antigens in the brain.

To test this possibility we made use of transgenic mice expressing a T cell receptor for a 1-11 peptide of MBP. Two types of such transgenic mice exist: mice bearing a transgene on a WT background ( $T_{MBP}$ ; these mice bear about 2% of endogenous T cells, which have a suppressive activity, namely regulatory T cells) or on a background of immune-deficient mice ( $RAG1^{-/-}$ ), therefore these animals lack the endogenous population of regulatory T cells. We exposed animals belonging to these two groups of mice to predator odor. Since these transgenic mice are on C57Bl/6J rather than BALB/c (**Fig. 1**), we examined in parallel C57Bl/6J WT animals.  $T_{MBP}$  mice showed a prevalence of PTSD similar to WT animals (40%), whereas  $T_{MBP}/Rag1^{-/-}$  mice were completely well-adapted to stress and no PTSD-like behavior was seen in these animals (**Fig. 2a**). These results suggested that perhaps the better ability of the  $T_{MBP}/Rag1^{-/-}$  mice to adapt to the stress relative to  $T_{MBP}$  mice was a reflection of the higher availability of autoimmune T cells in the former due to the absence of regulatory T cells. It is noted that SCID mice on this background (C57Bl/6J) showed a higher prevalence than their WT counterpart, similar to what was obtained with BALB/c/OLA SCID mice (**Fig. 1**).  $T_{MBP}/Rag1^{-/-}$  mice spent less time than other groups of mice in closed arms (**Fig. 2b**), whereas the startle response of  $T_{MBP}/Rag1^{-/-}$  mice was significantly higher than that of other groups of mice (**Fig. 2c**), however they underwent habituation (**Fig. 2d**).

### **Example 3. Depletion of $CD4^{+}CD25^{+}$ T cells increases the ability to cope with acute psychological stress**

The above results suggested that T cells directed to self-antigen are required to cope with psychological stress.  $T_{MBP}/Rag1^{-/-}$  and  $T_{MBP}$  mice, though to a lesser extent, develop with age autoimmune disease due to the uncontrolled levels of autoimmune T cells. Nevertheless, these experiments suggested that normal animals might benefit for fighting off stressful conditions, from a transient reduction in the activity of regulatory T cells. Naturally occurring Treg comprise approximately 10% of the  $CD4^{+}$  T-cell population (Shevach, 2000). These cells were shown to suppress ability to fight off degenerative conditions in the CNS imposed for

example by axonal injury (Kipnis et al., 2002a). To address the possibility that these cells control the spontaneous ability to fight off mental stress, we compared nude mice replenished with splenocytes obtained from WT mice depleted of Treg (devoid of Treg) to nude mice replenished with a normal splenocyte population (i.e., including Treg). In nude mice replenished with splenocytes free of Treg the prevalence of maladaptation was significantly lower (20%) than in the mice replenished with a normal T-cell population containing both Treg and effector T cells (50%) ( $\chi^2=4.0$ ,  $P<0.046$ ; (**Fig. 3a**). Significant differences between the two groups were observed both in the ASR (**Fig. 3b**) and in the time spent in the closed arms of the EPM (**Fig. 3c**).

#### **Example 4. T-cell accumulation in the brains of stressed mice correlates with behavioral adaptation**

In mice and rats suffering from neurodegenerative conditions, the beneficial effect of T cells is correlated with accumulation of T cells at the site of the lesion (Butovsky et al., 2001; Hauben et al., 2000a). To determine the relationship between the observed beneficial effect of the T-cell response and the consequences of exposure to stressful psychological conditions we examined whether it involved homing of T cells to the CNS. This was done by comparing the immunocytochemical staining for T cells in brain slices obtained from mice replenished with splenocytes depleted of Treg with those from mice replenished with a whole splenocyte population. Staining of brain slices with hematoxylin and eosin revealed no structural alterations in the hippocampus or amygdala (data not shown) between these two groups and the WT mice. Luxol fast blue staining for myelin reactivity also showed no differences between maladapted (**Figs. 4ai and 4aiii**) and well-adapted mice (**Fig. 4bi and 4biii**) compared to the WT (**Fig. 4ci**). Staining with anti-CD3 antibodies, however, revealed large numbers of T cells in these brain regions of well-adapted mice (**Fig. 4aii and 4aiv**) and hardly any in maladapted (**Figs. 4bii and 4biv**) or normal WT mice (**Fig. 4cii**), suggesting that the recruitment of T cells to the brain is correlated with the resistance to mental

stress. We wish, however, to emphasize that accumulation of T cells altogether is modest and by no means imply that PTSD can benefit from inflammation, rather that a well-controlled adaptive immunity assists in resisting consequences of mental stress, similarly to physical stress, and through the same mechanism.

5

## Discussion

The results of the present invention as described in section I above show that adaptation to acute psychological stressor of predator scent is dependent on autoimmune T cell-mediated response and is limited by naturally occurring  
10 CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Maladaptation after exposure to the stressor was significantly more prevalent in mice devoid of adaptive immunity than in WT mice with normal immune functioning. Transgenic mice expressing T cell receptor for MBP peptide (1-11) on a background of immune deficient mice did not show any maladaptation to stress. Moreover, the prevalence of maladapted responses was  
15 significantly reduced by depletion of the suppressive Treg in WT animals. Adaptation to stress was found to be correlated with accumulation of T cells in several brain areas.

T cells were recently shown to be involved in brain processes e.g., learning and memory. Deficiency in adaptive immunity led to cognitive dysfunction,  
20 whereas boost T cells cross-reactive with brain-specific antigens ameliorated symptoms of psychosis-like behavior induced by neurotransmitter imbalance (Kipnis et al., 2004). According to the concept of protective autoimmunity, in normal individuals the defense mechanisms involving anti-self immune cells are normally limited by naturally occurring Treg. These cells are formed during  
25 ontogeny and are viewed by immunologists as the cells that ensure that autoimmune T cell responses in the periphery will be kept in a state of tolerance. Studies have pointed out that defense against neurodegenerative conditions would benefit from elimination or weakening of these regulatory T cells (Kipnis et al., 2002a). According to the present invention, we show an additional aspect of defense that  
30 can enjoy removal or weakening of these naturally occurring Treg cells, namely,

coping with psychological stress. It thus appears that the very same mechanism that limits ability to cope with physical trauma to the CNS is responsible for the limited ability to cope with psychological stress.

5 The precise mechanism underlying the posttraumatic sequelae of psychological trauma is not fully known, nor is it clear whether or how neural death takes place under such conditions (McEwen, 1997). In the context of biochemical or physical insults to the CNS that lead to neuronal loss, the benefit of adaptive immunity is mediated via T cells that recognize self-antigens presented to them at the site of the insult and thereby allow their homing and locally activation. Once  
10 activated, such T cells ensure shaping of resident innate immune cells (i.e. microglia) to defense mode that could be tolerated by the delicate CNS tissue (Schwartz et al., 2003).

T cells that can endow mice with adaptation to stress are shown in the present invention to be directed against the brain-specific antigen MBP. Mice  
15 bearing T<sub>MBP</sub> on a background of immune deficient (RAG1<sup>-/-</sup>) mice, lack a subpopulation of endogenous regulatory T cells and thus develop spontaneous autoimmune diseases. These mice, which differ from SCID animals only in a monospecific population of autoimmune 1-11 MBP peptide specific T cells, did not develop any signs of anxiety following exposure to stress, which implies that if  
20 MBP reactive T cells are not the cells that mediate the spontaneous effect in wild type animals (T cells specific to other brain-restricted proteins might have similar effect), at least these are the cells that have the potential of doing such an antipsychotic effect. In the case of psychological stress, neither the precise location of the damaged area nor the identity of the antigens is known. Nevertheless, the  
25 observed salutary effect of depletion of Treg, in wild type animals, on the ability to contain adverse mental conditions, the ability to reject tumors, and the ability to withstand CNS injuries suggest that this T-cell subpopulation plays a key role in the cross-talk that regulates a fundamental part of the body's system of maintenance. These results thus emphasize that in normal healthy mice the compromise level of  
30 autoimmunity, determined by the absolute amount of such T cells and the relative

level of relevant regulatory T cells, needed for helping brain for coping with stress while avoiding autoimmune diseases is possibly sufficient for mild conditions. These findings further highlight the function of a properly operating immune system, in which the balance between the autoimmune and the naturally occurring regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells plays a pivotal role in maintaining the balance between the need for adaptive autoimmunity as a way of recruiting help to rescue tissues in distress and the need to avoid an unnecessary risk of autoimmune disease (Schwartz and Kipnis, 2002). To the best of our knowledge, this is the first demonstration that the peripheral adaptive immune system (represented preferentially by autoimmune T cells) affects the consequences of a psychologically traumatic experience. Therapeutic strategies based on re-charging peripheral immunity by vaccination with antigens that cross-react with a wide range of self-reacting T cells or by weakening regulatory T cells might provide ways for ensuring boosting peripheral immunity for CNS homeostasis under mental stress. Complete deprivation of animal from Treg will predispose it for spontaneous development of autoimmune diseases. Therefore, we would not suggest to utilize this method for development of anti-psychotic drugs for human use. However, partial and transient alleviation of suppression imposed by Treg might be of a powerful safe therapy for fighting off conditions leading to PTSD.

## SECTION II

### Down-regulation of Treg by dopamine and agonists

The present invention was undertaken in an attempt to identify physiological compounds potentially capable of controlling the Treg activity after CNS injury. We postulated that because stressor pain-related physiological compounds are increased after CNS injury (Rothblat and Schneider, 1998; Thiffault et al., 2000), one or more of them might transmit an early signal to Treg, with consequent reduction of the trafficking or suppressive activity, or both, of the latter. We reasoned that likely candidate compounds might be key neurotransmitters such as

dopamine, norepinephrine, serotonin, and substance P, all of which have been shown to participate in interactions between the brain and the immune system (Edgar et al., 2002).

Of all the tested neurotransmitters, dopamine was the only one that reduced the activity of Treg, and it did so via an ERK extracellular signal-regulated kinase-dependent pathway. Dopamine affected both the suppressive and the trafficking activities of Treg, via dopamine type 1 (D1-R and D5-R) receptors, found here to be preferentially expressed by Treg. Using mouse models of neurodegenerative conditions caused by partial crush injury of the optic nerve or glutamate intoxication in the eye, we showed that systemic administration of dopamine or its D1-type agonist can induce neuroprotection after mechanical and chemical CNS injury by alleviating the suppression imposed by Treg.

## Materials and Methods (Section II)

**(xii) *Animals.*** Inbred adult WT, SCID, and nu/nu BALB/c and C57Bl/6 mice were used in the experiments.

**(xiii) *Antibodies and reagents.*** Mouse recombinant IL-2, anti mouse  $\zeta$ -CD3 anti-mouse CTLA-4, and purified rabbit anti-mouse ERK2 antibody (R&D Systems, Minneapolis, Minnesota); rat anti-mouse phycoerythrin (PE)-conjugated CD25 antibody (PharMingen, Becton-Dickinson, Franklin Lakes, NJ); FITC-conjugated anti-CD4 antibody (Serotec, Oxford, UK); anti D1-R (Calbiochem, Darmstadt, Germany); 3-hydroxytyramine (dopamine), norepinephrine, SKF-38393, SCH-23390, quinpirole, clozapine, genistein, and PD98059 (Sigma-Aldrich, Rehovot, Israel); phosphatidyl serine detection kit (IQ Products, Houston, TX). Purified anti-pERK1/2 antibody was a gift from Prof. R. Seger (Weizmann Institute of Science).

**(xiv) *Intravitreal glutamate injection.*** The right eyes of anesthetized mice were punctured with a 27-gauge needle in the upper part of the sclera, and 10  $\mu$ l Hamilton syringe with a 30 gauge needle was inserted as far as the vitreal body. A total volume of 1  $\mu$ l of L-glutamate (400 nmol) dissolved in saline was injected into the eye (Schori et al., 2001).

(xv) ***Retrograde labeling of retinal ganglion cells (RGC).*** Mice were anesthetized and placed in a stereotactic device. The skull was exposed and kept dry and clean. The bregma was identified and marked. The designated point injection was at a depth of 2 mm from the brain surface, 2.92 mm behind the bregma in the anteroposterior axis, and 0.5 mm lateral to the midline. The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO) was applied (1  $\mu$ l, at a rate of 0.5  $\mu$ l/min in each hemisphere) using a Hamilton syringe, and the skin over the wound was sutured.

(xvi) ***Crush injury of the optic nerve in mice.*** Animals were anesthetized deeply by intraperitoneal injection of 2% Xyl-M (xylazine, 10 mg/kg; Arendonk, FF, Belgium) and Ketaset (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and subjected to severe crush injury of the intraorbital portion of the optic nerve. The uninjured contralateral nerve was left undisturbed. The optic nerve was crushed 3 d after retrograde labeling of retinal ganglion cells with FluoroGold, as described above (Fisher et al., 2001).

(xvii) ***Enzyme-linked immunosorbent assay.*** Treg or Teff ( $0.5 \times 10^6$  cells/ml) were cultured for 48 hr in the presence of anti-CD3 and anti-CD28. After 48 hr, the cells were centrifuged and their supernatants were collected and sampled. Concentrations of IL-2 in the samples were determined by the use of sandwich ELISA kits (R&D Systems). For detection of secreted IL-10, cells were centrifuged every 24 hr and replaced with a fresh medium. Supernatants obtained from cells after 24, 48, and 72 hr in culture were subjected to an ELISA kit (Diacclone Research, Fleming, France).

(xviii) ***Purification of murine  $CD4^+CD25^+$  and  $CD4^+CD25^-$  T cells*** was carried out as described in Materials and Methods, Section I (x).

(xix) ***T-cell adhesion.*** Adhesion of activated  $CD4^+CD25^+$  and  $CD4^+CD25^-$  T-cells to CSPG was analyzed as described previously (Ariel et al., 1998). Briefly, flat-bottomed microtiter (96-well) plates were precoated with CSPG (1  $\mu$ g/well, 40 min, 37°C).  $^{51}$ Cr-labeled T-cells were left untreated or were preincubated (30 min, 37°C) with dopamine or the specified agonist or antagonist ( $10^{-5}$ M). The cells ( $10^5$

cells in 100  $\mu$ l of RPMI medium containing 0.1% BSA) were then added to the CSPG-coated wells, incubated (30 min, 37°C), and washed. Adherent cells were lysed, and the resulting supernatants were removed and counted in a gamma counter. Results were expressed as the mean percentage of the total population before adhesion of bound T-cells from quadruplicate wells for each experimental group.

(xx) *Chemotaxis assay.* The migration of T-cells across polycarbonate filters (pore size, 5  $\mu$ m; diameter, 6.5 mm) toward SDF-1 and monocyte-derived chemokine (MDC/CCL22) was assayed in 24-well Transwell chambers (Costar, Corning, Corning, NY). T-lymphocytes ( $1.67 \times 10^6$  cells/ml) were suspended in RPMI medium/0.1% BSA, and 150  $\mu$ l of the cell suspension was added to the upper chamber after incubation with or without dopamine (90 min, 37°C). Chemokines were added to the lower chamber at concentrations of 1  $\mu$ g/ml SDF-1 (CytoLab, Israel) and 0.25  $\mu$ g/ml MDC (R & D Systems). The plates were incubated for 90 min at 37°C in 9.5% CO<sub>2</sub>. T-cells that migrated to the lower chambers were collected and stained with anti-CD4 and anti-CD25 antibodies. The numbers of migrating T-cells were measured by flow cytometer acquisition for a fixed time (60 sec). To calculate specific migration, the number of cells in each subpopulation in the absence of chemokine was subtracted from the number in the corresponding cell subpopulation that migrated in the presence of chemokines. The number of migrating CD4<sup>+</sup>CD25<sup>+</sup> T-cells was calculated as a percentage of the total T-cell population before migration. For migration of purified population, we used a similar protocol.

(xxi) *Activation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells.* Purified regulatory T cells (Treg;  $0.5 \times 10^6$ /ml) were activated in RPMI medium supplemented with L-glutamine (2 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml), non-essential amino acids (1 ml/100 ml), and autologous serum 2% (vol/vol) in the presence of mrIL-2 (5 ng/ml) and soluble anti-CD3 antibodies (1 ng/ml). Irradiated (2500 rad) splenocytes ( $1.5 \times 10^6$ /ml) were added to the culture. Cells were activated for 24 or 96 hr. In

some of the 96 hr experiments, fresh dopamine was added to the culture every 24 h during activation.

(xxii) **Inhibition assay (co-culturing of Teff with Treg).** Naïve effector T cells (Teff;  $50 \times 10^3$ /well) were co-cultured with decreasing numbers of activated  
5 Treg for 72 h in 96-well flat-bottomed plates in the presence of irradiated splenocytes ( $10^6$ /ml) supplemented with anti-CD3 antibodies. [ $^3$ H]-thymidine (1  $\mu$ Ci) was added for the last 16 hr of culture. After the cells were harvested, their analyzed [ $^3$ H]-thymidine content was analyzed by the use of a gamma counter.

(xxiii) **Immunocytochemistry.** T-cells were fixed for 10 min with a mixture  
10 (1:1) of methanol and acetone at  $-20^\circ\text{C}$ , incubated in blocking solution PBS containing 0.3% Triton-X100 and 1% of normal rabbit serum) for 60 min at room temperature, and then incubated overnight with a specific antibody (dilution, 1:1000) in the blocking solution. The T-cells were then washed and incubated with the secondary antibody (PE-labeled goat anti-rabbit IgG) for 30 min at room  
15 temperature, then washed, and analyzed by fluorescence and confocal microscopy.

(xxiv) **Western blotting.** Cells were stimulated for 20 min with anti-CD3 and anti-CD28 antibodies in the presence or absence of dopamine or the D1-R agonist SKF-38393. Cell lysates were prepared using radioimmunoprecipitation assay lysis buffer (50mM Tris, pH 8, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 500 mM  
20 NaCl, and 10 mM  $\text{MgCl}_2$ ). Supernatants were collected, and 5x sample buffer (containing 25 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 0.5M  $\beta$ -mercaptoethanol) was added before boiling. Activated ERK1/2 was detected by probing blots with a monoclonal antibody. Total ERK protein was detected by using a polyclonal rabbit antibody. The blots were developed by HRP-  
25 conjugated anti-mouse or anti-rabbit Fab and ECL (Amersham). Signals were quantified using NIH Image version 1.62.

(xxv) **Polymerase chain reaction (PCR).** Total RNA was purified with the RNeasy Mini Kit (Qiagen, Germantown, Maryland) and transcribed into cDNA using poly dT primers. For PCR, the following primers were used (for dopamine  
30 receptors primers were used from Lemmer et al., 2002);

D1-R: sense, 5'-GTAGCCATTATGATCGTCAC-3' (SEQ ID NO: 18), anti-sense, 5'-GATCACAGACAGTGTCTTCAG-3' (SEQ ID NO: 19);

D5-R: sense, 5'-CTACGAGCGCAAGATGACC-3' (SEQ ID NO: 20), anti-sense, 5'-CTCTGAGCATGCTCAGCTG-3' (SEQ ID NO: 21);

5 CCR-4: sense 5'-GTGCAGTCCTGAAGGACTTCAAGCTCCACCAG-3' (SEQ ID NO: 22), antisense 5'-GGCAAGGACCCTGACCTATGGGGTCATCAC-3' (SEQ ID NO: 23);

FOXP3: sense 5'-CAGCTGCCTACAGTGCCCCTAG-3' (SEQ ID NO: 24), antisense 5'-CATTTGCCAGCAGTGGGTAG-3' (SEQ ID NO: 25).

10 Signals were quantified using a Gel-Pro analyzer 3.1 (Media Cybernetics, Silver Spring, MD). Real-time PCR was performed with a LightCycler instrument (Roche, Mannheim, Germany) using the FastStart DNA Master SYBR Green 1 kit (catalog #3003230; Roche) as described by the manufacturer. The following primers were used:

15 D5-R: sense 5'-CCTTTATCCCGGTCCA-3' (SEQ ID NO: 26), antisense 5'-GATACGGCGGATCTGAA-3' (SEQ ID NO: 27);

IL-10: sense 5' ACCTGGTAGAAGTGATGCCCCAGGCA-3' (SEQ ID NO: 28), antisense 5'-CTATGCAGTTGATGAAGATGTCAAA-3' (SEQ ID NO: 29).

20

#### **EXAMPLE 5. Dopamine reduces the suppression imposed by Treg**

Coculturing of Teff with Treg isolated from naive mice results in suppression of Teff proliferation. The suppressive potency depends on the Treg/Teff ratio and the state of Treg activation; the suppression is significantly increased, for example, 25 if the Treg are activated before being added to Teff (Thornton and Shevach, 1998). Inhibition of Teff proliferation, assayed by [<sup>3</sup>H]-thymidine incorporation, can therefore be taken as a measure of the suppressive effect of Treg.

We examined the ability of major neurotransmitters and neuropeptides (dopamine, norepinephrine, substance P, and serotonin) to alleviate the Treg- 30 induced suppression of Teff *in vitro*. Each compound was tested at several

concentrations. Proliferation of Teff was significantly inhibited by cocultivation of Teff with naive Treg or with Treg that had been activated by incubation for 24 hr with anti-CD3 antibodies and IL-2 in the presence of antigen-presenting cells (APCs; lethally irradiated splenocytes) (**Fig. 5**). After incubating the activated Treg for 2 hr with a neurotransmitter or a neuropeptide, we washed the cells and then cocultured them with Teff. Proliferation of Teff cocultured with activated Treg that had been incubated with dopamine ( $10^{-5}$  M) was more than two-fold higher than proliferation in coculture with activated Treg not incubated with dopamine (**Fig. 5a**). A significant effect on Treg-suppressive activity was also obtained with  $10^{-7}$  M dopamine (**Fig. 5a**), whereas  $10^{-9}$  M had no significant effect (data not shown). The inhibitory effect of dopamine at  $10^{-5}$  and  $10^{-7}$  M on Treg activity was reproduced when freshly isolated (nonactivated) Treg were used (**Fig. 5b**). At the dopamine concentration of  $10^{-9}$  M, the obtained effect was slight and not statistically significant (**Fig. 5b**). It should be noted, however, that the effect of dopamine on Treg-suppressive activity was only partial and that complete blocking was not seen at any of the concentrations tested.

We also examined the effect of dopamine on the activity of Treg that had been activated as described above (**Fig. 5a**), but for 96 hr, and to which dopamine ( $10^{-5}$  M) was added for 2 hr at the end of the activation period and then washed off before the activated cells were cocultured with naive Teff. Again, Teff proliferation was significantly higher in the presence of activated Treg treated with dopamine than in the presence of activated Treg without dopamine (**Fig. 5c**). A direct effect of dopamine on Teff proliferation was ruled out by incubation of Teff for 2 hr with  $10^{-5}$  M dopamine, then washing off the dopamine and adding activated Treg without dopamine. The resulting proliferation of Teff did not differ from that seen in cultures of Teff in the absence of dopamine. Moreover, the inhibitory effects of Treg on naive Teff and on Teff exposed to dopamine were similar (**Fig. 5c**), indicating that dopamine did not alter the susceptibility of Teff to Treg suppression. The uptake of thymidine by Teff and the Treg-induced inhibition of such uptake varied from one experiment to another. In all experiments, however, the effect of

dopamine on Treg (tested >20 times) was consistent, and in most cases, the proliferation of Teff cocultured with Treg treated with dopamine was more than twofold higher than that in the absence of dopamine treatment. The Treg used in this experiment were always obtained from naive animals, therefore, it is unlikely that they contained any activated effector T-cells. The purity of the Treg population used in all experiments was high (between 92 and 98% of the total CD4 population). Moreover, the use of anti-CD25 antibodies to isolate Treg reportedly does not interfere with either the suppressive activity or the state of activation of Treg (Thornton and Shevach, 1998).

In contrast to the effect seen with dopamine, no effect on the ability of Treg to suppress Teff proliferation could be detected when Treg were preincubated with different concentrations of norepinephrine (another member of the catecholamine family) (**Fig. 6a**), substance P (a pain- and stress-related neurotransmitter; data not shown), or serotonin (data not shown).

#### **EXAMPLE 6. Dopamine (DA) acts on regulatory T cells (Treg) through its specific D1 receptor (D1-R)**

To establish whether the observed effect of dopamine on Treg is exerted through a receptor-mediated pathway, we used specific agonists and antagonists of dopamine receptors. Incubation of Treg with  $10^{-5}$  M SKF-38393, an agonist of the type 1 family of dopamine receptors (consisting of D1-R and D5-R), reproduced the dopamine effect (**Fig. 6b**). The specific D1-type antagonist SCH-23390 ( $10^{-5}$  M), when added together with dopamine ( $10^{-5}$  M), prevented the dopamine effect, further substantiating the contention that the effect of dopamine on Treg is mediated through the type 1 receptor family. Also in line with this contention was the finding that incubation of Treg with  $10^{-5}$  M quinpirole, an agonist of the type 2 family of dopamine receptors (comprising D2-R, D3-R, and D4-R), had no effect on the suppressive activity of Treg.

To exclude the possibility that dopamine exerts its effect by causing the death of Treg, we examined whether dopamine at the concentrations used here

cause Treg apoptosis. No signs of apoptosis were detectable in Treg, which, after being incubated with dopamine, were stained with propidium iodide and analyzed for apoptotic cells (sub-G1) by flow cytometry (**Fig. 6c**). To further verify the absence of apoptotic death in Treg, after incubating Treg with dopamine, we stained them for phosphatidylserine with annexin V. Again, we could not detect any signs of apoptosis in Treg beyond the background levels seen in the absence of dopamine (**Fig. 6d**). Thus, the reduction in Treg activity after their encounter with dopamine or a related agonist evidently results not from the death of Treg but rather from alteration of their behavior.

#### **EXAMPLE 7. Expression of dopamine receptors by Teff and Treg**

Because dopamine reduced the suppressive activity of Treg on Teff but did not alter the susceptibility of Teff to suppression by Treg, we examined the possibility that Teff and Treg express different subtypes or different amounts of the relevant dopamine receptors. This was done by assaying the expression of the dopamine type 1 receptors, D1-R and D5-R, in Treg and Teff. PCR assays showed that Treg expressed significantly more D1-R and D5-R transcripts (4-fold and 14-fold, respectively) than Teff (**Fig. 7a,b**). To further verify the differences in expression of dopamine receptors by Teff and Treg, we performed real-time PCR, which showed that the amounts of D1-R and D5-R in Treg were 5-fold and 13-fold higher, respectively, than in Teff (**Fig. 7c**).

We also used PCR to assay the expression of dopamine type 2 family receptors, namely D2-R, D3-R, and D4-R, in Treg and Teff. Although the expression of D4-R was somewhat more abundant in Teff than in Treg, the difference between the expression of each of these receptors in the two T-cell subpopulations was not significant (**Fig. 7d,e**). To verify that the difference in D1-R between Treg and Teff observed at the transcript level is manifested also at the protein level, we subjected the cells to immunocytochemical analysis. D1-R immunoreactivity was detected in naive Treg but not in naive Teff (**Fig. 7f**).

**EXAMPLE 8. Dopamine-mediated effect on Treg**

To gain additional insight into the mechanism whereby dopamine affects Treg activity, we examined CTLA-4, a molecule characteristic of Treg (Im et al., 2001). Expression of this molecule was slightly, but consistently, decreased on exposure of Treg to dopamine. A similar effect on CTLA-4 expression was obtained with the D1-type-specific agonist SKF-38393 (**Fig. 8a**). Another molecule that participates in the suppressive activity of Treg is IL-10 (Maloy et al., 2003). It was therefore of interest to measure the production of IL-10 by Treg after their exposure to dopamine. Media collected after incubation of Treg with dopamine ( $10^{-5}$  M) for 24, 48, and 72 hr showed a significant decrease in the amount of IL-10 at all time points examined (**Fig. 8b**). Dopamine did not, however, alter the anergic state of Treg; production of IL-2 was not detected in Treg that had been incubated in the presence of dopamine, as verified by ELISA for a secreted cytokine in media conditioned for 48 hr by activated Treg (**Fig. 8c**). Teff, as expected, secreted IL-2, the level of which was not affected by dopamine (**Fig. 8c**). It should be noted that activation of both T-cell populations was performed in the absence of mrIL-2.

A gene encoding the Foxp3 protein was recently found to be associated with Treg (Ramsdell, 2003). We therefore examined whether the dopamine-induced reduction of Treg activity alters the expression of this gene. mRNA isolated from Treg that were activated for 24 hr, exposed for 2 hr to dopamine, and maintained in culture for an additional 30 min or 24 hr was analyzed for Foxp3 expression. Foxp3, as expected, was detected in Treg, but no significant change in its expression was observed after Treg were exposed to dopamine for 30 min (**Fig. 8d**) or 24 hr (data not shown).

**EXAMPLE 9. ERK1/2 is deactivated by dopamine in Treg**

The finding that dopamine downregulated Treg activity via D1-type, but not D2-type, receptors, taken together with the recent report that the ERK pathway can be activated by D1-R dependent signaling (Takeuchi and Fukunaga, 2003), led us to

suspect that the downregulatory effect of dopamine on the suppressive activity of Treg might be exerted via the ERK pathway. To examine this possibility, we first treated Treg with the protein tyrosine kinase inhibitor genistein (4',5,7-trihydroxy isoflavone), which inhibits ERK and MEK (MAP/ERK kinase) activation (Mocsai et al., 2000). This treatment blocked the suppressive activity of Treg on Teff (**Fig. 9a**). Genistein at the same concentration had no effect on the proliferation of Teff (**Fig. 9a**).

In light of these results, we also examined whether Treg activity is affected by PD98059, a specific MEK inhibitor that blocks the ERK1/2 signaling pathway (Sharp et al., 1997). PD98059 significantly reduced the suppressive activity of Treg relative to that of control-activated Treg (**Fig. 9b**).

The above findings prompted us to examine the state of ERK phosphorylation in activated Treg in the presence or absence of dopamine. Treg were activated with anti-CD3 and anti-CD28 for 20 min in the presence or absence of dopamine ( $10^{-5}$ M), and Western blot analysis of phospho-ERK1/2 expression in lysates of Treg and Teff was performed. Significantly more phosphorylated ERK1/2 was detected in activated Treg than in activated Teff. Moreover, phospho-ERK1/2 was found to be downregulated in Treg that had been activated in the presence of dopamine (**Fig. 10a**). ERK1/2 phosphorylation in Treg was also reduced by the specific D1-type receptor agonist SKF (**Fig. 10b**). Results of the quantitative analysis of the phospho-bands are shown in **Figure 10c**.

#### **EXAMPLE 10. Dopamine alters the adhesive and migratory properties of Treg**

One of the main features of T-cells is their ability to migrate to tissues in need of rescue or repair [such as a diseased or damaged CNS (Hickey, 1999)]. We therefore considered the possibility that dopamine reduces not only the suppressive activity but also the migratory ability of Treg. Because T-cell migration and adhesion have been linked to ERK activation (Tanimura et al., 2003), this assumption appeared even more feasible in light of the above observation that dopamine reduced ERK activation in Treg. We incubated Treg with dopamine for 2

hr and then examined their adhesion to CSPG, extracellular matrix proteins often associated with injured tissues (Jones et al., 2003). The ability of Treg to adhere to CSPG was significantly greater than that of Teff (**Fig. 11a**) and was significantly decreased, in a concentration-dependent manner ( $10^{-9}$  to  $10^{-5}$  M), by dopamine (**Fig. 11a**). The dopamine effect on Treg could be mimicked by the D1-typespecific agonist SKF-38393 and inhibited by the D1-type antagonist SCH-23390. Dopamine had only a slight, nonsignificant effect on the adhesion of Teff to CSPG (**Fig. 11a**). The ability of Treg to adhere to fibronectin was greater than that of Teff (**Fig. 11b**). Exposure to dopamine resulted in no effect on adhesion of Treg to fibronectin and a slight increase in the adhesion of Teff (**Fig. 11b**).

To verify that the effect of dopamine on adhesion of Treg exerted through the ERK1/2 pathway, we incubated Treg with the ERK1/2 signaling pathway inhibitor PD98059 before performing the adhesion assay. PD98059 significantly reduced the ability of Treg to adhere to CSPG (**Fig. 11c**). Because interaction of T-cells with CSPG is mediated in part by the CD44 receptor (Henke et al., 1996), and in light of the known dependence of CD44 expression on the ERK signaling pathway, it was conceivable that dopamine might affect the expression of CD44 in Treg. To examine this possibility, we assayed CD44 immunoreactivity in Treg and Teff that had been activated with anti-CD3 and anti-CD28 antibodies for 24 hr and then incubated for 2 hr with or without dopamine. In the absence of dopamine, CD44 immunoreactivity was significantly stronger in Treg than in Teff. Dopamine decreased CD44 immunoreactivity in Treg but not in Teff (**Fig. 11d**). Other adhesion molecule receptors that we tested, such as leukotactic factor activity-1, intercellular adhesion molecule, and vascular cell adhesion molecule (Lee and Benveniste, 1999), did not show any dopamine-related changes in Treg (data not shown).

Migration of Treg in humans is dependent on the chemokine receptors CCR-4 and CCR-8, which are abundantly present on Treg (Sebastiani et al., 2001). We therefore examined whether exposure to dopamine would also affect Treg migration. For this experiment, we used a normal population of CD4<sup>+</sup> T-cells, of

which Treg (CD4<sup>+</sup>CD25<sup>+</sup>) accounts for ~11% (**Fig. 12a**). Of the CD4<sup>+</sup> cells that migrated toward CCL22 (MDC; a chemokine for CCR-4), 17% were Treg (CD4<sup>+</sup>CD25<sup>+</sup>), pointing to the greater migratory ability of Treg than of Teff toward MDC. However, after exposure of the CD4<sup>+</sup> cell population to dopamine, migrating  
5 Treg accounted for ~ 10% (the same as their percentage in the total CD4<sup>+</sup> population at the start of the experiment), suggesting that after their exposure to dopamine, Treg lost their preference for migration toward MDC.

We also examined the migration of a mixed T-cell population toward SDF-1. Migration of Teff toward SDF-1 was significantly greater than that of Treg  
10 (postmigration percentage of Treg in the total CD4<sup>+</sup> population was <4%), and dopamine did not alter this pattern (**Fig. 12a**). To examine the direct effect of dopamine on the migration of Treg, we assayed the effect of dopamine on the migration of purified Treg toward MDC. The migratory Treg were stained for CD4 to ensure that cell debris and aggregates would not be counted among them.  
15 Dopamine almost completely abolished Treg migration (**Fig. 12b,c**) but had no effect on the migration of Teff (data not shown).

In an attempt to link the changes in migration to specific receptors, we examined the expression of mRNA for CCR-4, CCR-8, and CXCR-4. Before the cells were exposed to dopamine, their CCR-4 expression, as expected from previous  
20 findings in human Treg (Sebastiani et al., 2001), was significantly higher in Treg than in Teff, but on exposure to dopamine, the expression of CCR-4 in Treg was decreased (**Fig. 12d,e**). The expression of mRNA encoding for CXCR-4 and CCR-8 did not change in Treg after these cells were exposed to dopamine (data not shown).

## 25 **EXAMPLE 11. Exogenous dopamine increases the ability to fight off neurodegeneration**

A previous study by our group showed that injection of activated Treg into mice (BALB/c) immediately after CNS injury significantly inhibits the spontaneous neuroprotective response, with the result that fewer neurons survive the  
30 consequences of the insult (Kipnis et al., 2002a). In the same study, we showed that

depletion of Treg increases the ability to withstand the insult. The present observation that Treg and Teff respond differentially to dopamine prompted us to examine the effect of dopamine on the ability to withstand neurotoxic conditions *in vivo*. We reasoned that systemic injection of dopamine after a CNS insult, by  
5 weakening the Treg activity, would improve recovery after a mechanical CNS injury. We subjected two groups of mice to a severe optic nerve crush injury and immediately thereafter gave injections of dopamine (0.4 mg/kg) to the mice in one group and injections of PBS to those in the other group. Two weeks later, their retinas were excised and neuronal survival was assessed. Significantly more viable  
10 neurons ( $1110 \pm 56/\text{mm}^2$ ; mean  $\pm$ SD) were found in the retinas of dopamine-injected mice than in the retinas of vehicle-treated mice ( $789 \pm 23$ ) (**Fig. 13a**).

We tested the beneficial effect of systemic dopamine in an additional model of neuronal degeneration induced by glutamate, a common player in many neurodegenerative conditions (Katayama et al., 1990; Xiong et al., 2003). Injection  
15 of glutamate into the eyes of adult mice causes retinal ganglion cell death that is measurable 1 week after the injection (Schori et al., 2001). We gave BALB/c mice intraperitoneal injections of dopamine, or its specific D1-type agonist SKF-38393 (3.3 mg/kg), or its specific D1-type antagonist SCH-23390 (3 mg/kg), immediately after their exposure to glutamate toxicity. We also gave SCID BALB/c mice  
20 injections of SKF-38393 (3.3 mg/kg) immediately after glutamate intoxication. A single systemic injection of dopamine (0.4 mg/kg) or its D1-type agonist given immediately after intraocular injection of a toxic dose of glutamate increased neuronal survival by  $18 \pm 2.5$  or  $19 \pm 3.2\%$ , respectively, relative to that in glutamate-injected controls treated with PBS (**Table 1**). Injection of the same  
25 agonist to scid mice resulted in no effect, thus supporting the assumption that systemic dopamine benefit CNS neurons via the peripheral immune system. The systemic injection of D1-type antagonist was done in an attempt to find out whether dopamine is involved, at least in part, in the spontaneous ability to withstand the insult. The injection of the antagonist resulted in a decrease in neuronal survival  
30 ( $11 \pm 1.5\%$ ;  $p < 0.01$ ) (**Table 1**) relative to that in PBS-injected mice, conceivably

because it competed with the endogenous dopamine for reduction of the suppressive activity of Treg after an injury. The above results suggested that dopamine might be one of the endogenous signals initiating the cascade that leads to spontaneous T-cell-dependent neuroprotection.

5 **Table 1. Neuronal survival after glutamate intoxication in mice given injections of dopamine or its type 1 receptor agonist and antagonist**

Mice	Treatment		
	Dopamine	SKF-38393	SCH-23390
Wild type	18 +2.5***	19 +3.2**	-11 +1.5**
SCID	NT	3+1.8(ns)	NT

10 Immediately after glutamate intoxication, mice were given systemic injections of the indicated drugs. Neuronal survival was determined 10 d later (see Materials and Methods). The results are expressed by changes (in percentage) in neuronal survival in treated mice relative to untreated mice. Each value represents a mean  $\pm$ SEM of a group at least five animals, and each experiment was performed at least twice, independently. Asterisks (\*\*\* $p$ <0.001; \*\* $p$ <0.01) indicate statistical significance of the presented data from a single experiment using a Student's  $t$  test  
 15 statistical analysis. NT, Not tested; ns, no statistical significance.

**EXAMPLE 12. Exposure of Treg to dopamine *in vitro* reduces their suppressive activity *in vivo***

20 The above results suggested that systemic dopamine can benefit injured CNS tissue in a T-cell-dependent pathway. To show this could be a consequence of a direct effect of dopamine on Treg activity *in vivo*, we examined whether dopamine can reduce the suppressive activity of Treg in an *in vivo* model of neuronal survival. Systemic injection of Treg after glutamate intoxication resulted in a 25% increase in  
 25 neuronal death. Incubation of Treg with dopamine before their systemic injection into mice abolished their suppressive effect, indicated by the lack of change in the number of surviving neurons. No effect on neuronal survival after glutamate intoxication could be detected in control mice given injections of Teff (**Fig. 13b**). **Figure 13c** shows representative micrographs of fields from retinas excised from

mice that were exposed to intravitreally injected glutamate and then injected with either Treg or Teff.

**EXAMPLE 13. Dopamine with the D2-R antagonist clozapine increase neuronal survival after glutamate-induced neuronal cell death**

Mice were injected intraocularly with a toxic dose of glutamate followed by an immediate injection i.v of the D2-R family antagonist clozapine (5 mg/kg) or with clozapine in combination with dopamine. Retinas were excised 7 days afterwards and survived neurons were counted. The results are depicted in **Fig. 14**.

Mice injected with clozapine alone showed a significant increase in neuronal survival compared to vehicle-injected mice. Moreover, mice injected with clozapine in combination with dopamine showed even higher neuronal survival.

**Discussion**

The results obtained in the above Examples 5-13 indicate that dopamine is a physiological compound that controls Treg activity on a daily basis and might provide an early spontaneous trigger for autoimmunity by targeting Treg and blocking their suppressive activity. Physiological activation of immunity against self, unlike immunity against non-self, apparently requires another signal in addition to the two signals (antigen recognition by T cells on MHC class II proteins, and co-stimulatory molecules) needed for activation of any T cell response to any antigen. The additional signal appears to be the rate-limiting one, which has the effect of releasing the autoimmune T cells (Teff) from the suppression that keeps them under control. We suggest that at least after injury to the CNS the additional signal is delivered by dopamine affecting Treg.

The above scenario exemplifies how the brain might control an autoimmune response, with dopamine as a short-lived physiological molecule enabling Treg to sense the need for autoimmunity. The site of dopamine interaction with Treg has yet to be discovered. We have previously shown that depletion of Treg in mice subjected to CNS insults improves neuronal survival, and that addition of Treg to

normal mice diminishes their ability to withstand CNS insults. It is therefore possible that, after damage to the CNS, a transient increase in dopamine can trigger the release of autoimmune T cells (Teff), which home to the site under stress.

Dopamine reduced Treg activity, and this was correlated with a decrease in  
5 ERK1/2 activation. In line with this observed correlation was the finding that adhesive and migratory abilities of Treg were reduced by dopamine via the ERK pathway.

Treg might exert their suppressive activity on Teff (autoimmune T-cells) either in the lymphoid organs or at the site of the neural tissue degeneration.  
10 Mediation of the suppressive activity of Treg has been attributed partially to IL-10 and CTLA-4, whereas their migration and adhesion have been attributed to the specific repertoire of chemokine receptors and adhesion molecules that they express (Sebastiani et al., 2001). Reduction of the suppressive activity of Treg was correlated with a decrease in their IL-10 production (Zhang et al., 2004) and CTLA-  
15 4 expression, which might participate in the cytokine-mediated and cell-cell-mediated suppression by Treg, respectively. Moreover, Treg express relatively large amounts of the CD44 receptor (needed for their adhesion to CSPG) and the chemokine receptor CCR-4 (needed for their migratory ability). The exposure of Treg to dopamine resulted in a decrease in both their adhesion to CSPG and their  
20 migration toward MDC, in correlation with their diminished expression of CD44 and CCR-4, respectively.

We found that significantly more D1-R and D5-R are expressed by Treg than by Teff. The marked difference in D1-R and D5-R expression, which is hardly detectable on Teff or any other immune cells (Ricci et al., 1997), makes the D1-type  
25 receptor family a likely candidate for the dialog of dopamine with Treg, leading, via the ERK pathway, to reduction of the suppressive activity of Treg. It is interesting to note that D2-R, which antagonizes D1-R, activates ERK (Pozzi et al., 2003).

We found that the effect of dopamine on the suppressive activity of Treg was weak compared with the effect of a protein tyrosine kinase inhibitor such as  
30 genistein (Mocsai et al., 2000) or the ERK1/2 signaling pathway inhibitor PD98059,

indicating that dopamine is a suitable candidate as a physiological immunomodulator mainly in the context of autoimmune activity.

Treg exist in a state of anergy, neither proliferating in response to mitogenic stimuli nor producing IL-2. Although dopamine downregulated the suppressive activity of Treg, it did not reverse the anergic state of these T-cells with respect to proliferation or IL-2 production, supporting the contention that dopamine induces changes in the activity rather than in the phenotype of Treg.

The *in vivo* relevance of the effect of dopamine on the suppressive activity of Treg was demonstrated in the experimental paradigms of mouse optic nerve mechanical crush injury and glutamate intoxication in the mouse eye. Significantly more neurons survived consequences of optic nerve crush or neurotoxic insult in mice given injections of dopamine or its D1-type agonist. The observed lack of effect of the D1-type agonist SKF-38393 in mice devoid of mature T-cells substantiated our conclusion that the effect of peripheral dopamine on neuronal survival is exerted via the immune system and not directly on neural tissue. Moreover, dopamine, when injected systemically, does not cross the blood–brain barrier. That the *in vivo* effect of dopamine is through Treg was further demonstrated by the passive transfer of Treg after their exposure to dopamine. Treg suppressed the ability to resist neurodegeneration, as indicated by an increased loss of neurons. Incubation of Treg with dopamine before their transfer wiped out their suppressive effect on neuronal survival. The loss of Treg activity *in vivo* might reflect the effect of dopamine both on homing of Treg to the damaged site and on their suppression. The potential ability of endogenous dopamine to operate spontaneously *in vivo* was demonstrated by the decrease in neuronal survival in mice given injections of the D1-type antagonist SCH-23390 immediately after glutamate intoxication. The weak (11%) effect of SCH-23390 on neuronal survival appears to be attributable, at least in part, to the nature of the experimental model (Kipnis et al., 2002). The 11% decrease observed in the wild-type mice represents >30% of the maximal possible T-cell-dependent effect (the difference between nude and wild type). It is also possible that dopamine is a member of a

family of physiological compounds capable of controlling Treg activity after a CNS insult.

Previous studies have documented the effect of dopamine on T-cell adhesion (Levite et al., 2001), on activation (Ilani et al., 2001), and on T-lymphocyte suppression of IgG production by peripheral blood mononuclear cells (Kirtland et al., 1980). No attempt was made in any of those studies to attribute the dopamine effect to subpopulations of CD4<sup>+</sup> T-cells. Our results suggest that dopamine has a direct and preferential effect on Treg in initiating the immune response.

In light of the observed effect of dopamine on Treg according to the present invention, the uncontrolled presence of a stress signal such as dopamine might explain the aberrant immunity in patients with mental disorders (such as schizophrenia) associated with large amounts of dopamine, whereas deficiency in dopamine (as in Parkinson's disease) might explain accelerated neuronal loss.

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### SECTION III

#### Low-dose $\gamma$ -irradiation promotes survival of injured neurons in the CNS

##### Material and Methods – Section III

(xxvi) *Animals*. Inbred adult female Lewis and Sprague-Dawley (SPD) rats and BALB/c/OLA and C57Bl/6J wild type, *nu/nu*, and SCID mice were used in the experiments.

(xxvii) *Antigens*. MBP from guinea pig spinal cord was purchased from Sigma-Aldrich (St. Louis, MO).

(xxviii) *Antibodies*. Mouse anti-rat CD4 antibodies conjugated to phycoerythrin (PE) and mouse anti-rat CD25 antibodies conjugated to fluorescein isothiocyanate were purchased from Serotec (Oxford, UK). Rat anti-mouse PE-conjugated CD25 antibody (PC61) was purchased from Pharmingen (Becton-Dickinson, Franklin Lakes, NJ).

(xxix) *Crush injury of the optic nerve in rats and mice*. The optic nerve was crushed as previously described in detail (Yoles and Schwartz, 1998). Using a

binocular operating microscope, we anesthetized the animals and exposed their right optic nerves. In rats, we used calibrated cross-action forceps to inflict a moderate or severe crush injury on the optic nerve, 1–2 mm from the eye. The severity of the injury determines the number of directly injured neurons. To assess neuroprotection we inflicted a moderate crush injury on the optic nerve in Lewis and SPD rats (severe crush in Lewis rats leaves almost no viable retinal ganglion cells (RGCs) because of poor endogenous neuroprotection). Mice were subjected to severe crush injury to the intraorbital portion of one optic nerve (Fisher et al., 2001). In all cases, the contralateral nerve was left undisturbed.

(xxx) *Measurement of secondary neuronal degeneration in rats.* Secondary degeneration of optic nerve axons was assessed by retrograde labeling of RGCs. This was done by the application, 2 weeks after crush injury, of the fluorescent lipophilic dye 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Leiden, The Netherlands) distally to the site of lesion, as previously described (Yoles and Schwartz, 1998).

(xxxi) *Retrograde labeling of surviving retinal ganglion cells in mice.* In the mouse model, unlike in the rat, we assayed survival of RGCs and not secondary degeneration of neurons that escaped the primary lesion. The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO) was injected into the anesthetized mouse (1 µl, at a rate of 0.5 µl/min in each hemisphere) using a Hamilton syringe, at a depth of 2 mm from the exposed brain surface, 2.92 mm posterior to the bregma and 0.5 mm lateral to the midline. One week after crush injury the mice were killed and their retinas were detached and prepared as flattened whole mounts in 4% paraformaldehyde solution. Labeled cells from 4 to 6 selected fields of identical size (0.7 mm<sup>2</sup>) were counted (Fisher et al., 2001).

(xxxii) *Spinal cord injury.* Rats were anesthetized by intraperitoneal injection of Rompun (xylazine, 10 mg/kg; Vitamed, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and their spinal cords were exposed by laminectomy at the level of T8. One hour after induction of anesthesia, we dropped a 10-g rod onto the laminectomized cord from a height of 50 mm

(“severe” injury) or 25 mm (“mild” injury), using the NYU impactor, a device shown to inflict a well-calibrated contusive injury of the spinal cord (Hauben et al., 2000a, 2000b; Basso et al., 1996; Young, 1996).

(xxxiii) *Assessment of recovery from spinal cord contusion.* Functional recovery was determined by locomotor hindlimb performance, which was scored using the BBB open field locomotor rating scale of Basso, Beattie, and Bresnahan, on a scale of 0 (complete paralysis) to 21 (normal mobility) (Hauben et al., 2001a; Basso et al., 1996). Blind scoring ensured that observers were not aware of the treatment received by each rat or mouse. Approximately once a week we evaluated the locomotor activities of the trunk, tail, and hind limbs in an open field by placing the animal for 4 min in the center of a circular enclosure (90 cm diameter, 7 cm wall height) made of molded plastic with a smooth, nonslip floor. Prior to each evaluation the animal was examined carefully for perineal infection, wounds in the hind limbs, and tail and foot autophagia. Behavioral data were analyzed by two-tailed Student’s t-tests. Open-field motor scores, measured at different times after the injury, were analyzed using two-factor repeated-measures ANOVA.

(xxxiv) *Animal care.* In spinally injured rats and mice, bladder expression was assisted by massage at least twice a day (particularly during the first 48 h after injury, when it was done up to three times a day), until the end of the 2<sup>nd</sup> week, by which time automatic voidance had been recovered. Animals were carefully monitored for evidence of urinary tract infection or any other sign of systemic disease. During the 1<sup>st</sup> week after contusion and in any case of hematuria after this period, they received a course of sulfamethoxazole (400 mg/ml) and trimethoprim (8 mg/ml) (Resprim, Teva Laboratories, Israel), administered orally with a tuberculin syringe (0.3 ml of solution per day). Daily inspections included examination of the laminectomy site for evidence of infection and assessment of the hind limbs for signs of autophagia or pressure.

(xxxv) *Preparation of splenocytes.* Donor splenocytes from rats (up to 10 weeks of age) were obtained by rupturing the spleen and following conventional

procedures. The splenocytes were washed with hypotonic buffer (ACK) to lyse red blood cells.

(xxxvi) *Purification of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells.* Regulatory (CD4<sup>+</sup>CD25<sup>+</sup>) T cells were purified according to a previously described procedure (Thornton and Shevach, 1998). Briefly, splenocytes and lymph node cells were passed through mouse CD3<sup>+</sup> columns (MTCC-25; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Purified T cells (CD3<sup>+</sup>) were incubated with anti CD8 antibody conjugated to magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After depletion by autoMACS the negative fraction (consisting of CD4<sup>+</sup> T cells) was incubated with anti-CD25 antibody conjugated to PE (Becton-Dickinson), washed, and incubated with anti-PE antibody conjugated to magnetic beads (Miltenyi Biotec) and positively selected by autoMACS. The positive fraction was tested by FACSsort (Becton-Dickinson). Purity was in excess of 98%.

(xxxvii) *Depletion of CD25<sup>+</sup> cells.* Splenocytes prepared by a conventional procedure were incubated with rat anti-mouse PE-conjugated CD25 antibody, and this was followed by incubation with anti-PE beads (Becton-Dickinson). After being washed, the splenocytes were subjected to AutoMacs (Miltenyi Biotec) with the "deplete sensitive" program. Recovered populations were analyzed by FACSsort.

(xxxviii) *Cell cycle.* After purifying the T cells as described above, we fixed them with 70% ethanol for 20 min at -20°C and then applied Ribonuclease A (Sigma-Aldrich, Rehovot, Israel) for 2 h at -20°C. The cells were then washed, and after application of propidium iodide (PI; Sigma-Aldrich) they were analyzed by FACSsort.

(xxxix) *Polymerase chain reaction (PCR).* Total RNA was purified with the RNeasy Mini Kit (Qiagen, Germany) and transcribed into cDNA using poly dT primers. For PCR the following primers were used:

INF- $\gamma$  (product 405 bp): 5'-ATGAGTGCTACACGCCGCGTCTTGG-3' (SEQ ID NO: 30) and 3'-GAGTTCATTGACTTTGTGCTGG-5' (SEQ ID NO: 31);

IL-12 (product 386 bp): 5'-AGATGACATCACCTGGACCT-3' (SEQ ID NO: 32) and 3'-CTTTGGTTCAGTGTGACCTTC-5' (SEQ ID NO: 33);

IL-2R (product 423 bp): 5'-TGCCACGTTCAAAGCCCTCTCCTA-3' (SEQ ID NO: 34) and 3'-TGCGTCCACCTTATCTCCCCACAC-5' (SEQ ID NO: 35);

5 L19 (product 194 bp): 5'-CTGAAGGTCAAAGGGAATGTG-3' (SEQ ID NO: 36) and 3'-GGACAGAGTCTTGATGATCTC-5' (SEQ ID NO: 37).

The following examples 14-17 were carried out to examine whether the beneficial effect of low-dose  $\gamma$ -irradiation on neuronal survival after CNS injury is exerted through the immune system. Our results showed, as in the case of immune system participation in fighting off tumor growth (Safwat, 2000), that low-dose  $\gamma$ -irradiation causes induction of a strong immune response and production of proinflammatory cytokines. The immune activation is an outcome of the activation of effector T cells together with selective fatal effects of the irradiation on suppressive T cells. We found that rats or mice that were subjected to a single low dose of  $\gamma$ -irradiation recovered better from mechanical injury (optic nerve axotomy or spinal cord contusion) or biochemical insult (local glutamate toxicity) than matched controls not subjected to irradiation. Replenishment of  $\gamma$ -irradiated mice with activated Treg wiped out their advantage in terms of ability to withstand neuronal toxicity.

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**EXAMPLE 14. Low-dose total body or total lymphoid organ  $\gamma$ -irradiation in rats protects CNS neurons from injurious conditions.**

We first examined whether low-dose total body irradiation (TBI) of rats subjected to traumatic CNS injury increases neuronal survival. SPD rats received a single dose (350 rad) of TBI immediately after a crush injury to the left optic nerve. Neuronal survival (presented here as the mean number of surviving RGCs/mm<sup>2</sup>  $\pm$  SEM; *P* values were calculated by Student's *t*-test), assessed 2 weeks after the injury, was significantly higher in irradiated rats (72.8  $\pm$  8.6) than in nonirradiated controls (49.1  $\pm$  6; *P* < 0.01; Fig. 15A). When similarly injured Lewis rats (in which the spontaneous ability to manifest a protective autoimmunity is limited relative to

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that in SPD rats) were subjected to TBI, neuronal survival after optic nerve injury was increased to an even greater extent, by more than 3 fold ( $114.4 \pm 5.6$  compared to  $40.3 \pm 1.8$ ;  $P < 0.001$ ; **Fig. 15B**). A difference of this magnitude has significant biological meaning, as it implies rescue of approximately 3500 RGCs in the whole retina.

To verify that the beneficial effect of TBI on neuronal survival is an outcome of the effect of irradiation on the immune system and not on the damaged tissue itself, we used Lewis rats that had been thymectomized at birth and were therefore devoid of mature T cells as adults. Single-dose TBI (350 rad) immediately after optic nerve injury in thymectomized rats had no effect on neuronal survival ( $36 \pm 4$  compared to  $40.6 \pm 7$  in controls; **Fig. 15C**). These results support our suggestion that the beneficial effect of TBI on neuronal survival under neurodegenerative conditions is an outcome of the effect of irradiation on the systemic immune response, and is possibly mediated by T cells.

#### **EXAMPLE 15. Low-dose total body irradiation improves spontaneous recovery from spinal cord injury**

To determine whether TBI is beneficial in another model of acute axonal injury we subjected rats to severe spinal contusion, as previously described (Hauben et al., 2000a). We made sure that rats were matched by weight and age, so that the primary loss due to the mechanical insult would be of equal severity in all rats (Hauben et al., 2000a). Any differences in recovery would then reflect the change in secondary loss as a result of TBI. Assessment of recovery was based on locomotion in an open field measured by the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1996), in which a value of 0 represents complete paralysis and a value of 21 represents complete mobility. Two days after the injury the rats were randomly divided into two groups; one group was subjected to TBI and the other was left untreated. Hind limb motor performance was significantly better in the irradiated than in the untreated rats. Analysis by two-tailed T-test showed significant differences, starting from day 19 after the injury and at every time point

tested thereafter (**Fig. 16**). Mean scores  $\pm$  SEM after 52 days were  $7.7 \pm 0.05$  for irradiated and  $4.3 \pm 0.8$  for nonirradiated rats ( $P < 0.05$ , two-tailed  $t$ -test). This difference has significant biological meaning, as the motor score is nonlinear and thus an average value of 7.7 represents rats that can move all of their hind limb joints and walk (albeit without coordination), whereas rats with a BBB value of 4 and below drag their hind limbs (Hauben et al., 2000a, 2000b).

**EXAMPLE 16. Low-dose  $\gamma$ -irradiation leads to immune activation through homeostasis-driven proliferation of adaptive immunity.**

In light of previous studies by our group showing that Treg suppress the spontaneous ability to manifest a protective autoimmunity, we first examined whether Teff and Treg differ in their sensitivity to  $\gamma$ -irradiation. Each of these two cell populations was purified and activated for 48 h with anti-CD3 and anti-CD28 antibodies. Each was then subjected to  $\gamma$ -irradiation (350 rad) and left for an additional 48 h in the stimulation medium. At the end of the incubation period the cells were washed and stained with propidium iodide (PI) for detection of dead cells. The percentage of dead cells in the Treg population ( $68.4 \pm 5.6\%$ ) was significantly higher than in the Teff population ( $23.3 \pm 2.5\%$ ; **Fig. 17A**). No differences in cell death were seen between non-irradiated Teff and Treg. *In-vivo* examination of the spleens revealed a decrease of 2 to 3 fold in size as a result of the TBI (**Fig. 17B**). On the basis of the above results, we postulated that the TBI-induced ability to withstand the consequences of a CNS insult is due to an increase in the incidence of activated T cells among the remaining lymphocytes. To test this hypothesis we examined the activation state of the T cells 5 days after irradiation. TBI induced a significant increase in the numbers of  $CD4^+$  T cells expressing the CD25 marker (activated T cells) in the spleen, lymph nodes, and blood relative to nonirradiated  $CD4^+$  T cells (**Fig. 17C**). This finding was further substantiated by the observed increase in CD25 mRNA 3 days and 7 days after irradiation (**Fig. 17D**). An increase in CD25-expressing cells could result from activation of Teff or an

increase in the incidence of Treg. Since, as shown above, Treg are more sensitive than Teff to the  $\gamma$ -irradiation, the later possibility is very unlikely.

We further verified the irradiation-induced activation of T cells by comparing the expression of the proinflammatory cytokines IFN- $\gamma$  and IL-12 in T cells purified from splenocytes and lymph nodes of irradiated rats with that expressed by T cells isolated from nonirradiated rats. Expression of IFN- $\gamma$  was found to be increased 3 days after TBI and decreased 1 week later, whereas IL-12 expression was still significantly increased 1 week after TBI (Figs. 17E, 17F).

**EXAMPLE 17. Neuroprotection mediated by lymphopenia-induced activation of T cells in mice is diminished by injection of naturally occurring Treg.**

Since glutamate is among the most common mediators of neuronal degeneration in the CNS, we also examined the beneficial effect of TBI in a model of glutamate toxicity. After intravitreal injection of a toxic dose of glutamate (400 nmol), significantly more RGCs survived in mice that were subjected, immediately after the glutamate injection, to TBI (350 rad) than in nonirradiated mice (mean number of RGCs  $\pm$  SD mm<sup>2</sup>; 2709  $\pm$  51 compared to 1692  $\pm$  47;  $P < 0.001$ ). The beneficial effect of TBI decreased as the time interval between irradiation and the glutamate injection increased, suggesting that synchrony between proliferation and antigen presentation, both induced by the insult, is needed for the increase in the subpopulation of T cells specific to the insult-associated antigen. Thus, compared to nonirradiated controls (1736  $\pm$  65), the irradiated mice could still withstand the effects of glutamate toxicity injected 6 days after TBI (2366  $\pm$  94;  $P < 0.01$ ) and as late as 14 days after TBI (2037  $\pm$  70;  $P < 0.05$ ; **Fig. 18A**). To verify that the effect of irradiation in mice is immune mediated, we subjected SCID mice (which are totally devoid of T cells) to TBI (350 rad) after exposure of their RGCs to glutamate toxicity. No differences were observed between the irradiated and nonirradiated groups (1696  $\pm$  87 compared to 1788  $\pm$  62; **Fig. 18A**). Changes in neuronal survival are expressed as a percentage of the number of viable neurons in matched controls that were subjected to the insult but received no treatment.

In light of these results, we considered the possibility that  $\gamma$ -irradiation of the total lymphoid organs (TLI) might be as effective as TBI in inducing lymphopenia. To test this possibility, we subjected BALB/c mice to a single low-dose  $\gamma$ -irradiation (350 rad) of the lymphoid organs, followed immediately by intravitreal injection of a toxic dose of glutamate. Significantly more RGCs survived in mice subjected to TLI than in nonirradiated mice ( $2205 \pm 37$  compared to  $1910 \pm 35$ ;  $P < 0.001$ ; **Fig. 18B**). In the figure, the increase in RGC survival is expressed as a percentage of the number of viable RGCs in matched controls that were subjected to the insult but received no treatment.

To verify the apparent correlation between the beneficial effect of irradiation on neuronal survival and the proliferation of T cells as a result of the irradiation-induced depletion of Treg, we examined whether the effect of the irradiation is neutralized by the passive transfer of Treg ( $CD4^+CD25^+$  isolated from naïve matched WT mice). We subjected BALB/c mice to TBI and 3 days later exposed their RGCs to a toxic dose of intravitreal glutamate. One group of mice was then immediately injected with Treg and a second (control) group was injected with activated Teff ( $CD4^+CD25^-$  isolated from naïve matched WT mice (**Fig. 18C**). Neuronal survival was increased by approximately 30% after TBI, and Treg injection reduced this benefit to only 10%. Injection of Teff did not alter the TBI-induced beneficial effect on neuronal survival.

## SECTION IV

### Poly-YE effect on Treg

As mentioned hereinbefore, Poly-YE was described in WO 03/002140 of the present applicants as a neuroprotective agent capable of preventing or inhibiting neuronal degeneration in the CNS or PNS, for promoting nerve regeneration in the CNS or PNS, or for protecting CNS or PNS cells from glutamate toxicity. It was

therefore of interest to examine in established models as described above whether poly-YE will be beneficial for treatment of mental disorders.

#### Materials and Methods (Section IV)

5            *(xl) Animals.* Inbred adult male C57Bl/6J mice (8–12 weeks old) were housed in a light- and temperature-controlled room and matched for age in each experiment.

*(xli) Drug solutions.* Poly-YE (Poly (Glu,Tyr, 1:1, molecular weight 20,000-40,000, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS. Fresh solutions  
10 of dizocilpine maleate (MK-801; Sigma-Aldrich) were prepared in physiological saline (0.9% NaCl in sterile distilled water) for each batch of mice. Mice were injected with MK-801 or vehicle (PBS) 15 min before being subjected to behavioral tests.

*(xlii) Antibodies and reagents.* Mouse recombinant IL-2 (mrIL-2) and anti-  
15 mouse  $\zeta$ -CD3 (anti-CD3; clone 145-2C11) were purchased from R&D Systems (Minneapolis, MN, USA). Rat anti-mouse PE-conjugated CD25 antibody (PC61) was purchased from Pharmingen (Becton-Dickinson, Franklin Lakes, NJ, USA).

*(xliii) Preparation of lymphocytes.* Donor mice lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) were ruptured through  
20 mesh. The lymphocytes were washed with hypotonic buffer (ACK) to lyse red blood cells.

*(xliv) Preparation of splenocytes* was carried out as in Materials and Methods, Section III(xxxv).

*(xlv) Purification of  $CD4^+CD25^+$  and  $CD4^+CD25^-$  T cells.* Lymph nodes  
25 were harvested and mashed. T cells were enriched by negative selection and purified on CD3-cell columns (MTCC-25; R&D Systems). The enriched T cells were incubated with anti-CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and negatively selected  $CD4^+$  T cells were incubated with PE-conjugated anti-CD25 ( $30 \mu\text{g}/10^8$  cells) in PBS/2% fetal calf serum. They were then  
30 washed and incubated with anti-PE microbeads (Miltenyi Biotec) and subjected to

magnetic separation with AutoMACS (Miltenyi Biotec). The retained cells were eluted from the column as purified  $CD4^+CD25^+$  cells. The negative fraction consisted of  $CD4^+CD25^-$  T cells. Cell purity was checked by FACSsort (Becton-Dickinson) and typically ranged from 88% to 95%. Purified cells were cultured in 24-well plates (1 ml).

(xlvii) *Activation of  $CD4^+CD25^+$  regulatory T cells.* was carried out as in Materials and Methods, Section II(xxi). Cells were activated for 24 or 96 hours. In some of the experiments, poly-YE (20  $\mu$ g/ml) was added to the culture every 24 h during activation.

(xlviii) *Inhibition assay (co-culturing of Teff with Treg)* was carried out as in Materials and Methods, Section II(xxii).

(xlix) *Morris water maze (MWM) behavioral test.* Spatial memory was assessed by performance on the Morris water maze task, a hippocampal-dependent visuo-spatial learning task. Mice were given four trials per day, for 4 consecutive days, to find the hidden platform located 1.5 cm below the water surface in a pool 1.4 m in diameter. Within the testing room only distal visuo-spatial cues were available to the mice for location of the submerged platform. The escape latency, i.e., the time required by the mouse to find and climb onto the platform, was recorded for up to 60 s. Each mouse was allowed to remain on the platform for 30 s, and was then moved from the maze to its home cage. If the mouse did not find the platform within 120 s, it was manually placed on the platform and returned to its home cage after 30 s. The inter-trial interval was 30 s. On day 5 the platform was removed from the pool, and each mouse was tested by a probe trial for 60 s. On days 6–7 the platform was placed at the opposite location, and the mouse was retrained in four sessions. Data were recorded using an EthoVision automated tracking system (Noldus).

#### **EXAMPLE 18. Poly-YE alleviates the suppressive activity mediated by Treg**

Naïve Teff cells ( $50 \times 10^3$  cells/well) were co-cultured with decreasing numbers (50, 25, 12.5 and  $6.5 \times 10^3$  cells/well) of Treg cells that have been activated

for 24 h with anti-CD3 and mrIL-2. The activation of the Treg cells was carried out in the absence of poly-YE (control) or, after 24 h, activated Treg cells were incubated for 2 h with poly-YE (20 µg/ml in PBS) before co-culturing them with Teff (TregYE). To some of the co-cultures of Teff and Treg, poly-YE (20 µg/ml) was added (TregYE+YE) and the co-cultures were further incubated. **Figs. 19A-19B** show that incubation of the activated Treg for 2 h with poly-YE prior to their co-culturing with Teff (TregYE) alleviated the Treg suppressive activity on Teff, as measured by the resulting proliferation of Teff, compared to that obtained with activated Treg not exposed to poly-YE (control). **Fig. 19B** shows that the effect was even more significant in the co-cultures of Teff and TregYE to which poly-YE was added (TregYE+YE) as shown by the significantly higher Teff proliferation. The proliferation of Teff also increased with decreasing concentrations of activated Treg. T cell proliferation was assayed by incorporation of [<sup>3</sup>H]-thymidine into effector T cells co-cultured with Treg. Recorded values are from one representative experiment out of three and are expressed as means ± SD of 4 replicates.

#### **EXAMPLE 19. Poly-YE causes changes in the cytokine phenotype of Treg cells**

To test the cytokine profile of Treg cells upon incubation with poly-YE, Treg cells were incubated with mrIL-2 and anti-CD3 for 72 hours, washed and further incubated for 48 hours using fresh medium and poly-YE (20 µg/ml in PBS). Conditioned media were collected 24 hours after incubation with poly-YE and analyzed for cytokines using commercial kits for INF-γ, IL-10, TGF-β and IL-2, according to the manufacturer's instructions (all kits from R&D Systems, Biotest Ltd., Kfar Saba, Israel).

**Figs. 20A-20D** show that the cytokine phenotype of the T regulatory cells is changed in the presence of poly-YE and it becomes similar to the phenotype of T effector cells, namely, there is up regulation of IFN-γ (**Fig. 20A**), TGF-β (**20B**) and IL-2 (**Fig. 20C**) and down regulation of IL-10 (**Fig. 20D**). The change of the cytokine secretion profile of the Treg cells after incubation with poly-YE reflects the changes induced by poly-YE in the biological activity of the Treg cells. The

inhibitory cytokine IL-10 secretion is significantly reduced while secretion of the pro-inflammatory cytokine INF- $\gamma$  is elevated. The appearance of IL-2 secretion goes in line with the observation of increased proliferation of the Treg cells in the presence of poly-YE.

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**EXAMPLE 20. Poly-YE immunization is protective against cognitive impairment induced by psychotomimetic agents**

Since poly-Glu,Tyr was shown in Example 18 above to alleviate the suppressive activity of the Treg cells, we have then tested its effect in an animal model of psychotic behavior that simulates schizophrenia.

Dizocilpine maleate (+)MK-801, an antagonist of the N-methyl-D-aspartate (NMDA) receptor channel) act as psychotomimetic agents, inducing - via neurotransmitter imbalance - psychotic symptoms in healthy individuals and exacerbating psychotic symptoms in schizophrenic patients. We therefore used this compound in an animal model to induce psychotic behavior that simulates behavioral abnormalities associated with schizophrenia.

Administration of MK-801 also induces cognitive deficits in the mice. Numerous authors have reported an MK-801-induced learning deficit in acquisition of spatial memory (Whishaw and Auer, 1989; Ahlander et al., 1999) and non-spatial memory tasks (Griesbach et al., 1998). We therefore examined the effect of poly-YE immunization on the ability to prevent or reverse the cognitive deficit induced by MK-801.

One week before administration of MK-801, each mouse was either immunized with poly-YE (25  $\mu$ g/mouse subcutaneously) or with vehicle (PBS), and then injected intraperitoneally (i.p.) with MK-801 (0.1 mg/kg) 15 min before the mouse was tested.

Administration of MK-801 significantly impaired performance of a spatial memory task in the Morris water maze (MWM). **Fig. 21** depicts representative tracks of MK-801-injected poly-YE-immunized mice and of MK-801-injected PBS-injected control mice when tested in the MWM at the first day (trails 1 and 4). As

shown in **Fig. 22**, the swimming strategies of the poly-YE-immunized mice (left panels) and the PBS-treated controls (right panels) differed: the poly-YE-immunized mice employed more methodical swimming strategies than the controls. Thus, all of the poly-YE-immunized mice learned to swim away from the wall to search for the platform in the inner 50% of the pool and to use the platform as a refuge when they found it. In contrast, the behavior of the PBS-immunized mice showed severe disturbances, including hyperactivity, swimming over the platform, and aimless swimming in circles.

Injection of MK-801 significantly impaired task acquisition in the MWM (increased escape latency) in the PBS-treated mice, but not in poly-YE-immunized mice (**Fig. 22**). During the acquisition phase of the MWM task, the PBS-treated mice took significantly longer than the poly-YE-immunized mice to acquire the spatial navigation task, if they were able to acquire it at all. The poly-YE-immunized mice learned to swim to the hidden platform and make use of it as a refuge by climbing onto it and remaining there, as indicated by decreasing latencies in successive trials. In contrast, when the PBS-treated mice encountered the hidden platform they behaved in an abnormal and maladaptive way. Even when placed directly on the hidden platform after a trial in which they had failed to locate it, these mice quickly walked or jumped off and continued swimming in a haphazard and disorganized manner.

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**CLAIMS:**

1. A method for treatment of an individual suffering from a psychiatric disorder which comprises administering to said individual in need of such a treatment an effective amount of an agent that causes down-regulation of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells on CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (Teff), modulation of the immune response and/or modulation of autoimmune response, but excluding Copolymer 1, a Copolymer 1-related peptide and Copolymer 1-related polypeptide, and further excluding a combination of dopamine, a dopamine precursor or an agonist of the dopamine receptor type 1 family (D1-R agonist) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist), when said individual is one suffering from schizophrenia.
2. A method according to claim 1 wherein said agent that causes down-regulation of the Treg cells is low dose whole-body or total lymphoid organ  $\gamma$ -irradiation.
3. A method according to claim 1 wherein said agent that causes down-regulation of the Treg cells is selected from the group consisting of: (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) a combination of dopamine and a dopamine precursor; and (v) a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with an antagonist of the dopamine receptor type 2 family (dopamine D2-R antagonist) or a pharmaceutically acceptable salt thereof, provided that when said agent is said combination of (v), said individual is not one suffering from schizophrenia.
4. A method according to claim 3 wherein said agent is dopamine or a pharmaceutically acceptable salt thereof.

5. A method according to claim 3 wherein said agent is a combination of dopamine with the dopamine precursor levodopa, optionally in further combination with carbidopa.
6. A method according to claim 3 wherein said agent is a dopamine D1-R agonist selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine.
7. A method according to claim 3 wherein said agent is a combination of dopamine with a dopamine D2-R antagonist.
8. A method according to claim 7 wherein dopamine is in combination with a dopamine D2-R antagonist selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.
9. A method according to claim 3 wherein said agent is a combination of a dopamine D1-R agonist with a dopamine D2-R antagonist.
10. A method according to claim 9 wherein said agent is a combination of a dopamine D1-R agonist selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine, and a dopamine D2-R antagonist selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.
11. A method according to claim 1 wherein said agent causes modulation of the autoimmune response.

12. A method according to claim 11 wherein said agent is a modified central nervous system (CNS) peptide obtained by modification of a self-peptide derived from a CNS-specific antigen, which modification consists in the replacement of one or more amino acid residues of the self-peptide by different amino acid residues, said modified CNS peptide still being capable of recognizing the T-cell receptor  
5 recognized by the self-peptide but with less affinity.

13. A method according to claim 12 wherein said peptide is obtained by modification of a self-peptide derived from a CNS-specific antigen selected from the group consisting of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors.  
10

14. The method according to claim 13 wherein said modified CNS peptide is obtained by modification of a self-peptide selected from the group consisting of p11, p51-70, p87-99, p91-110, p131-150, and p151-170 of MBP.  
15

15. The method according to claim 14 wherein said modified MBP peptide has a sequence selected from the SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.

16. The method according to claim 13, wherein said CNS-specific antigen is a mammalian Nogo or Nogo receptor molecule and said peptide has a sequence selected from the SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:10 to NO:17.  
20

17. The method according to claim 11 wherein said agent that causes modulation of the autoimmune response are T cells that have been activated either by a CNS-specific antigen selected from the group consisting of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors, or by a modified peptide obtained by modification of a self-peptide derived from said CNS-specific antigen.  
25

18. The method according to claim 1 wherein said agent is poly-YE, a poly-YE related peptide or polypeptide.

19. The method according to claim 18 wherein said agent is poly-YE.

20. A method according to any one of claims 1 to 19 wherein said psychiatric  
5 disorder is selected from: (i) anxiety disorders, that include phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder,  
10 schizophreniform disorder, schizoaffective disorder and delusional disorder; (iv) drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, and phencyclidine use; and (v) memory loss disorders such as amnesia or memory loss associated with Alzheimer's type dementia or with non-Alzheimer's type dementia,  
15 e.g. multi-infarct dementia or memory loss associated with Parkinson's disease, Huntington's disease, Creutzfeld-Jakob disease, head trauma, HIV infection, hypothyroidism and vitamin B12 deficiency.

21. The method according to claim 20 wherein said psychiatric disorder is schizophrenia.

20 22. The method according to claim 20 wherein said psychiatric disorder is an anxiety disorder such as stress or post-traumatic stress disorder.

23. The method according to claim 20 wherein said psychiatric disorder is a mood disorder such as depression or a bipolar disorder.

24. A pharmaceutical composition for treatment of psychiatric disorders  
25 comprising a pharmaceutically acceptable carrier and an agent selected from the group consisting of: (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist

of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) a combination of dopamine and a dopamine precursor; (v) a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with an antagonist of the dopamine receptor type 2 family (dopamine D2-R antagonist) or a pharmaceutically acceptable salt thereof; (vi) a modified central nervous system (CNS) peptide obtained by modification of a self-peptide derived from a CNS-specific antigen; (vii) T cells that have been activated either by a CNS-specific antigen selected from the group consisting of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors, or by a modified peptide obtained by modification of a self-peptide derived from said CNS-specific antigen; and (viii) poly-YE, a poly-YE related peptide or polypeptide, provided that when said agent is said combination of (v), said individual is not one suffering from schizophrenia.

25. The pharmaceutical composition according to claim 24 wherein said agent is dopamine or a pharmaceutically acceptable salt thereof.

26. The pharmaceutical composition according to claim 24 wherein said agent is a combination of dopamine with its precursor levodopa, optionally in further combination with carbidopa.

27. The pharmaceutical composition according to claim 26 wherein dopamine, levodopa and carbidopa, if present, is each in a separate container.

28. The pharmaceutical composition according to claim 24 wherein said agent is a dopamine D1-R agonist.

29. The pharmaceutical composition according to claim 28 wherein said dopamine D1-R agonist is selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine.

30. The pharmaceutical composition according to claim 24 wherein said agent is a combination of dopamine with a dopamine D2-R antagonist.

31. The pharmaceutical composition according to claim 30 wherein said dopamine D2-R antagonist is selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.

32. The pharmaceutical composition according to claim 24 wherein said agent is a combination of a dopamine D1-R agonist with a dopamine D2-R antagonist.

33. The pharmaceutical composition according to claim 32 wherein said agent is a combination of a dopamine D1-R agonist selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine, and a dopamine D2-R antagonist selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2 as defined in any one of claims 3 to 10, excluding a combination of dopamine, a dopamine precursor or an agonist of the dopamine receptor type 1 family (D1-R agonist) with an antagonist of the dopamine receptor type 2 family (D2-R antagonist) for treatment of schizophrenia.

34. The pharmaceutical composition according to claim 24 wherein said agent is poly-YE, a poly-YE related peptide or polypeptide.

35. A pharmaceutical composition for treatment of psychiatric disorders comprising a pharmaceutically acceptable carrier and poly-YE.

36. A vaccine for immunization of an individual suffering from a psychiatric disorder comprising poly-YE.

37. A pharmaceutical composition according to any one of claims 24 to 36 wherein said psychiatric disorder is selected from: (i) anxiety disorders, that include phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder, schizophreniform disorder, schizoaffective disorder and delusional disorder; (iv) drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, and phencyclidine use; and (v) memory loss disorders such as amnesia or memory loss associated with Alzheimer's type dementia or with non-Alzheimer's type dementia, e.g. multi-infarct dementia or memory loss associated with Parkinson's disease, Huntington's disease, Creutzfeld-Jakob disease, head trauma, HIV infection, hypothyroidism and vitamin B12 deficiency.

38. The pharmaceutical composition according to claim 37 wherein said psychiatric disorder is schizophrenia.

39. The pharmaceutical composition according to claim 37 wherein said psychiatric disorder is an anxiety disorder such as stress or post-traumatic stress disorder.

40. The pharmaceutical composition according to claim 37 wherein said psychiatric disorder is a mood disorder such as depression or a bipolar disorder.

41. Use of an agent selected from the group consisting of: (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist of the dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) a combination of dopamine and a dopamine precursor; (v) a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with an antagonist of the dopamine receptor type 2 family (dopamine D2-R antagonist) or a

pharmaceutically acceptable salt thereof; (vi) a modified central nervous system (CNS) peptide obtained by modification of a self-peptide derived from a CNS-specific antigen; (vii) T cells that have been activated either by a CNS-specific antigen selected from the group consisting of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors, or by a modified peptide obtained by modification of a self-peptide derived from said CNS-specific antigen; and (viii) poly-YE, a poly-YE related peptide or polypeptide, for the preparation of a pharmaceutical composition for treatment of psychiatric disorders, but excluding a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with a dopamine D2-R antagonist for treatment of schizophrenia.

42. The use according to claim 41 wherein said agent is dopamine or a pharmaceutically acceptable salt thereof.

43. The use according to claim 41 wherein said agent is a combination of dopamine with its precursor levodopa, optionally in further combination with carbidopa.

44. The use according to claim 43 wherein dopamine, levodopa and carbidopa, if present, is each in a separate container.

45. The use according to claim 41 wherein said agent is a dopamine D1-R agonist.

46. The use according to claim 45 wherein said dopamine D1-R agonist is selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine.

47. The use according to claim 41 wherein said agent is a combination of dopamine with a dopamine D2-R antagonist.

48. The use according to claim 47 wherein said dopamine D2-R antagonist is selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.

49. The use according to claim 41 wherein said agent is a combination of a dopamine D1-R agonist with a dopamine D2-R antagonist.

50. The use according to claim 49 wherein said agent is a combination of a dopamine D1-R agonist selected from the group consisting of SKF-82958, SKF-38393, SKF-77434, SKF-81297, A-77636, fenoldopam and dihydrexidine, and a dopamine D2-R antagonist selected from the group consisting of amisulpride, eticlopride, raclopride, remoxipride, sulpride, tropapride, domperidone, iloperidone, risperidone, spiperone, haloperidol, spiroperidol, clozapine, olanzapine, sertindole, mazapertine succinate, zetidoline, CP-96345, LU111995, SDZ-HDC-912, and YM 09151-2.

51. The use according to claim 41 wherein said agent is poly-YE, a poly-YE related peptide or polypeptide.

52. Use of poly-YE for the preparation of a pharmaceutical composition for treatment of psychiatric disorders.

53. The use according to claim 52 wherein said composition is a vaccine for immunization,

54. The use according to any one of claims 41 to 53 wherein said psychiatric disorder is selected from: (i) anxiety disorders, that include phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder,

schizophreniform disorder, schizoaffective disorder and delusional disorder; (iv) drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, and phencyclidine use; and (v) memory loss disorders such as amnesia or memory loss  
5 associated with Alzheimer's type dementia or with non-Alzheimer's type dementia, e.g. multi-infarct dementia or memory loss associated with Parkinson's disease, Huntington's disease, Creutzfeld-Jakob disease, head trauma, HIV infection, hypothyroidism and vitamin B12 deficiency.

10 55. The use according to claim 54 wherein said psychiatric disorder is schizophrenia.

56. The use according to claim 54 wherein said psychiatric disorder is an anxiety disorder such as stress or post-traumatic stress disorder.

57. The use according to claim 54 wherein said psychiatric disorder is a mood disorder such as depression or a bipolar disorder.

15 58. An article of manufacture comprising packaging material and a pharmaceutical composition contained within the packaging material, said pharmaceutical composition comprising an agent selected from the group consisting of: (i) dopamine or a pharmaceutically acceptable salt thereof; (ii) a dopamine precursor or a pharmaceutically acceptable salt thereof; (iii) an agonist of the  
20 dopamine receptor type 1 family (D1-R agonist) or a pharmaceutically acceptable salt thereof; (iv) a combination of dopamine and a dopamine precursor; (v) a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with an antagonist of the dopamine receptor type 2 family (dopamine D2-R antagonist) or a pharmaceutically acceptable salt thereof; (vi) a modified central nervous system  
25 (CNS) peptide obtained by modification of a self-peptide derived from a CNS-specific antigen; (vii) T cells that have been activated either by a CNS-specific antigen selected from the group consisting of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated

glycoprotein (MAG), Nogo, Nogo receptor, S-100,  $\beta$ -amyloid, Thy-1, P0, P2, and neurotransmitter receptors, or by a modified peptide obtained by modification of a self-peptide derived from said CNS-specific antigen; and (viii) poly-YE, a poly-YE related peptide or polypeptide; and said packaging material includes a label that indicates that said agent is therapeutically effective for treating a psychiatric disorder, but excluding a combination of dopamine, a dopamine precursor or a dopamine D1-R agonist with a dopamine D2-R antagonist for treatment of schizophrenia.

59. An article of manufacture comprising packaging material and a pharmaceutical composition contained within the packaging material, said pharmaceutical composition comprising poly-YE, and said packaging material includes a label that indicates that said agent is therapeutically effective for treating a psychiatric disorder,

60. The article of manufacture according to claim 58 or 59 wherein said psychiatric disorder is selected from: (i) anxiety disorders, that include phobic disorders, obsessive-compulsive disorder, post-traumatic stress disorder (PTSD), acute stress disorder and generalized anxiety disorder; (ii) mood disorders, that include depression, dysthymic disorder, bipolar disorders and cyclothymic disorder; (iii) schizophrenia and related disorders such as brief psychotic disorder, schizophreniform disorder, schizoaffective disorder and delusional disorder; (iv) drug use and dependence such as alcoholism, opiate dependence, cocaine dependence, amphetamine dependence, hallucinogen dependence, and phencyclidine use; and (v) memory loss disorders such as amnesia or memory loss associated with Alzheimer's type dementia or with non-Alzheimer's type dementia, e.g. multi-infarct dementia or memory loss associated with Parkinson's disease, Huntington's disease, Creutzfeld-Jakob disease, head trauma, HIV infection, hypothyroidism and vitamin B12 deficiency.

61. The article of manufacture according to claim 61 wherein said psychiatric disorder is schizophrenia.

62. The article of manufacture according to claim 61 wherein said psychiatric disorder is an anxiety disorder such as stress or post-traumatic stress disorder.

5 63. The article of manufacture according to claim 61 wherein said psychiatric disorder is a mood disorder such as depression or a bipolar disorder.

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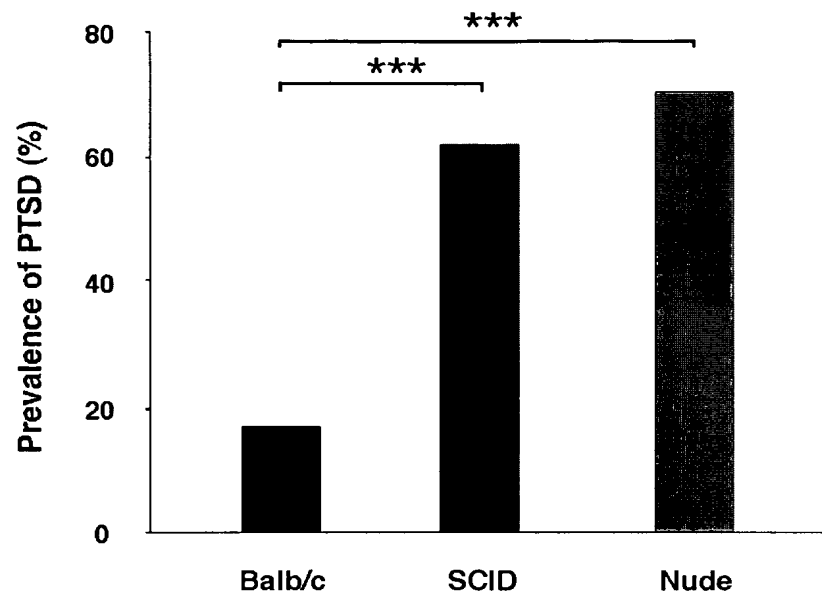


Fig. 1a

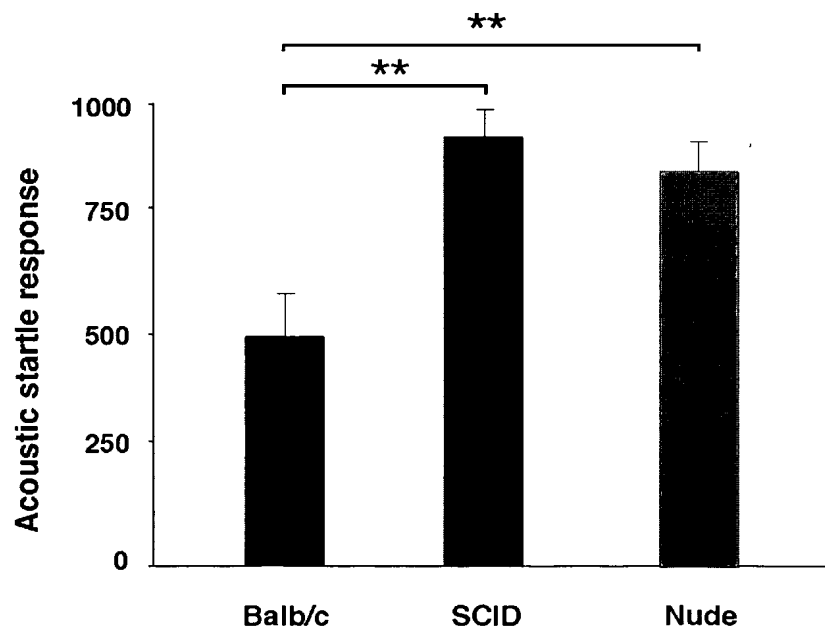


Fig. 1b

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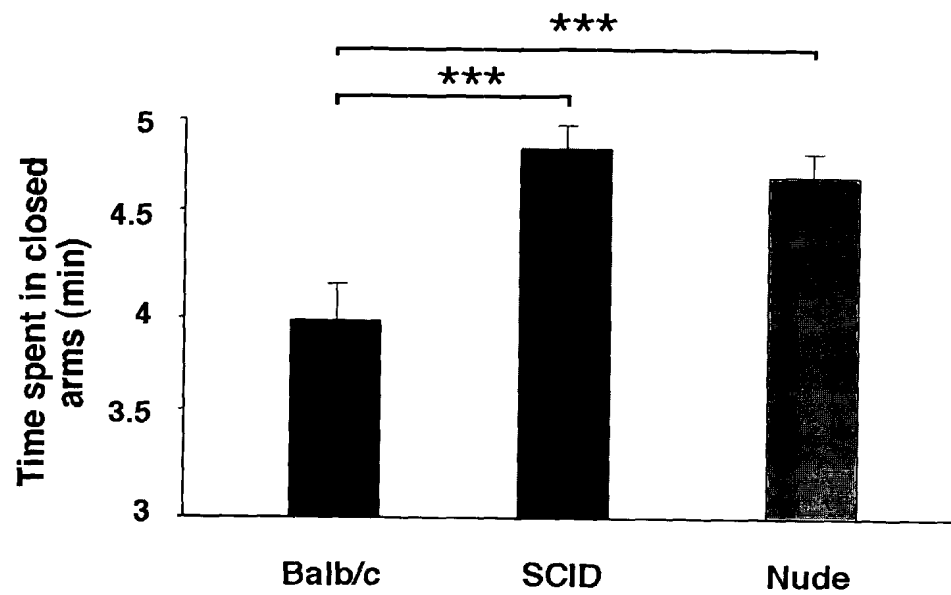


Fig. 1c

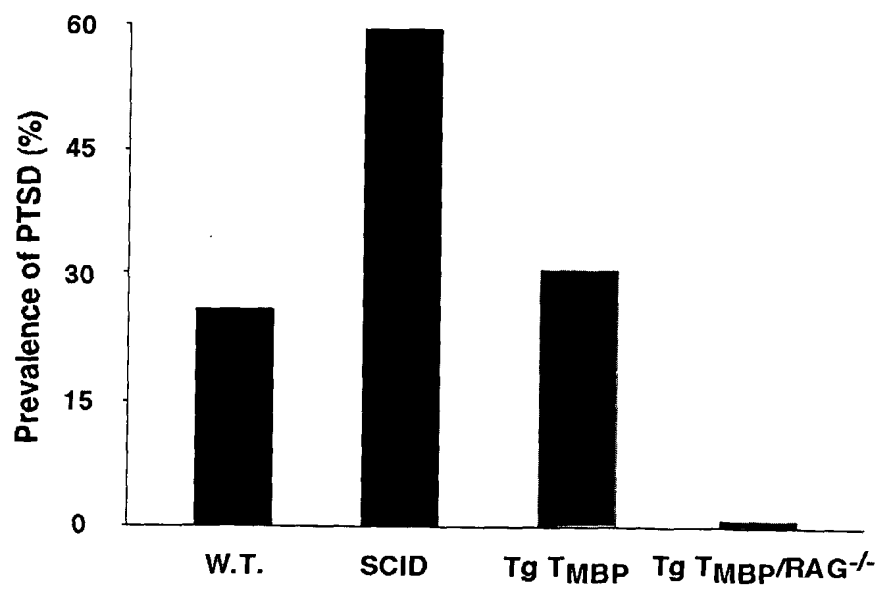


Fig. 2a

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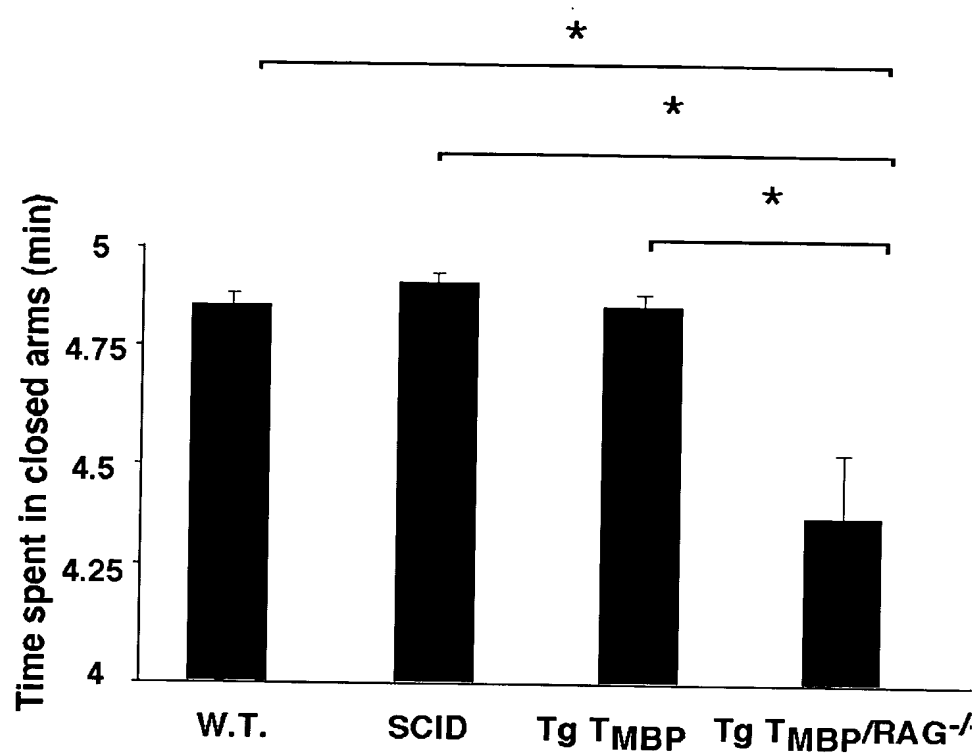


Fig. 2b

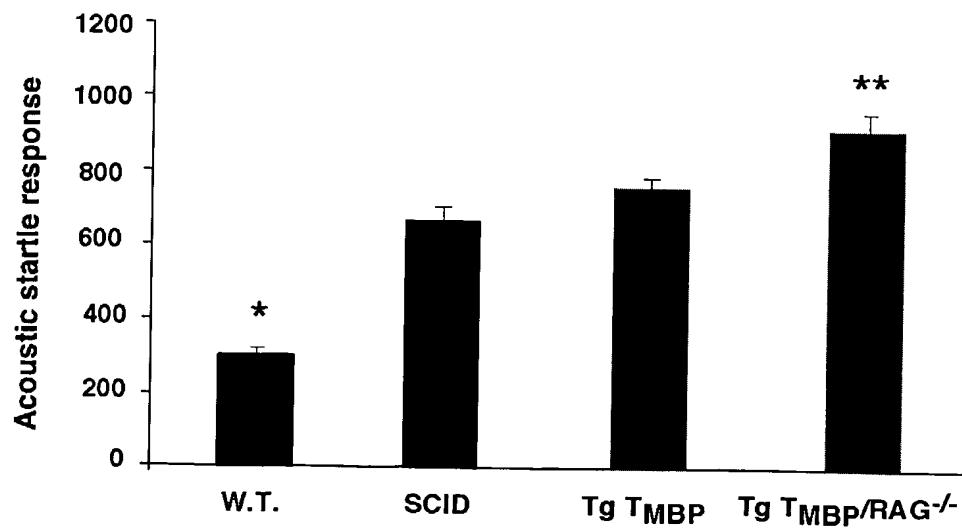


Fig. 2c

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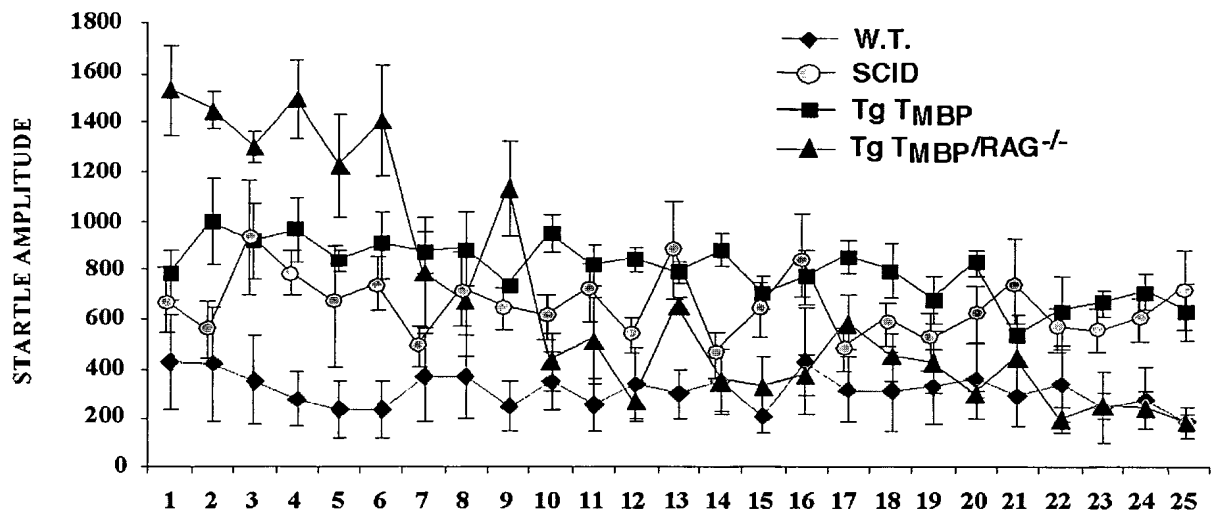


Fig. 2d

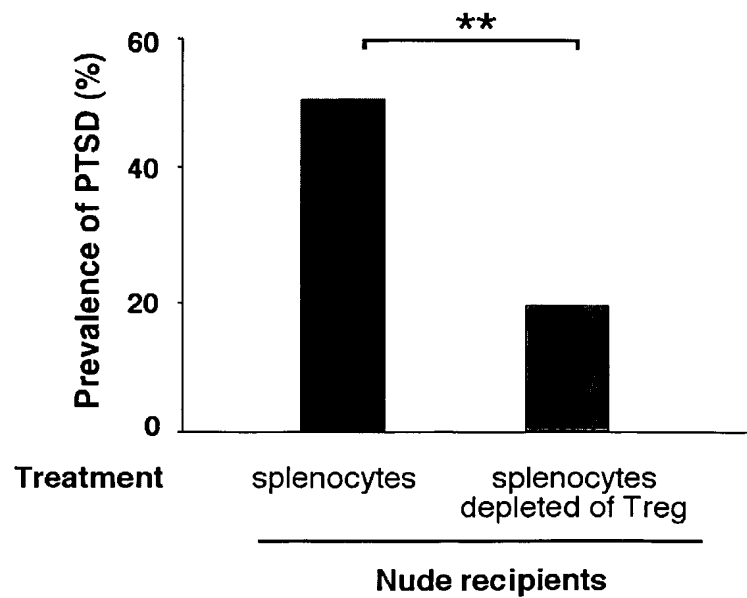


Fig. 3a

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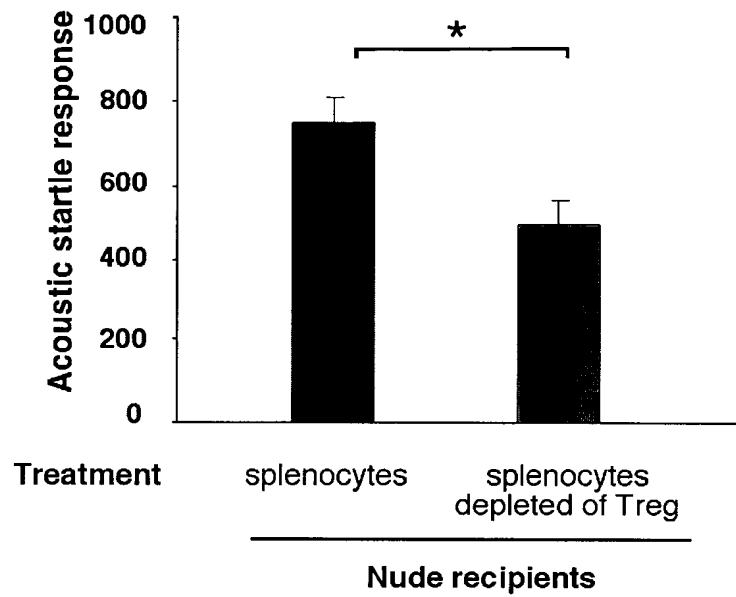


Fig. 3b

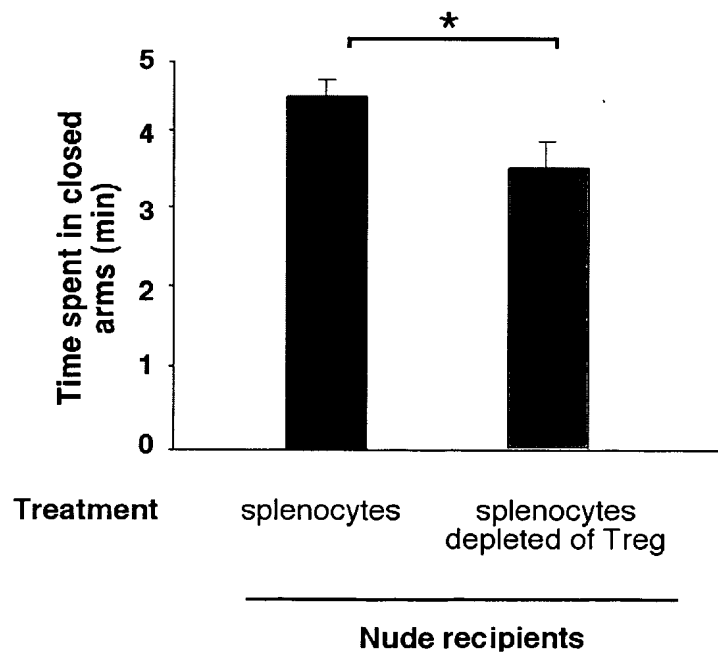
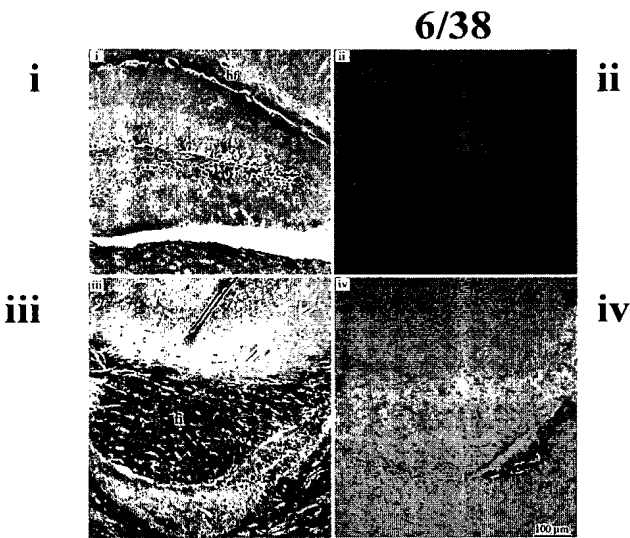
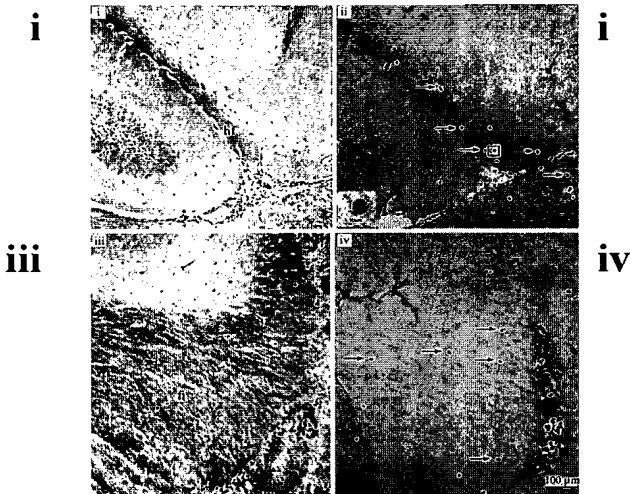


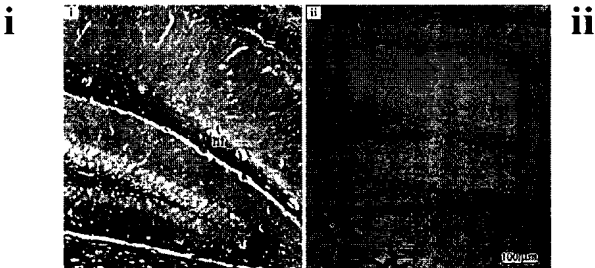
Fig. 3c



**Fig. 4a**



**Fig. 4b**



**Fig. 4c**

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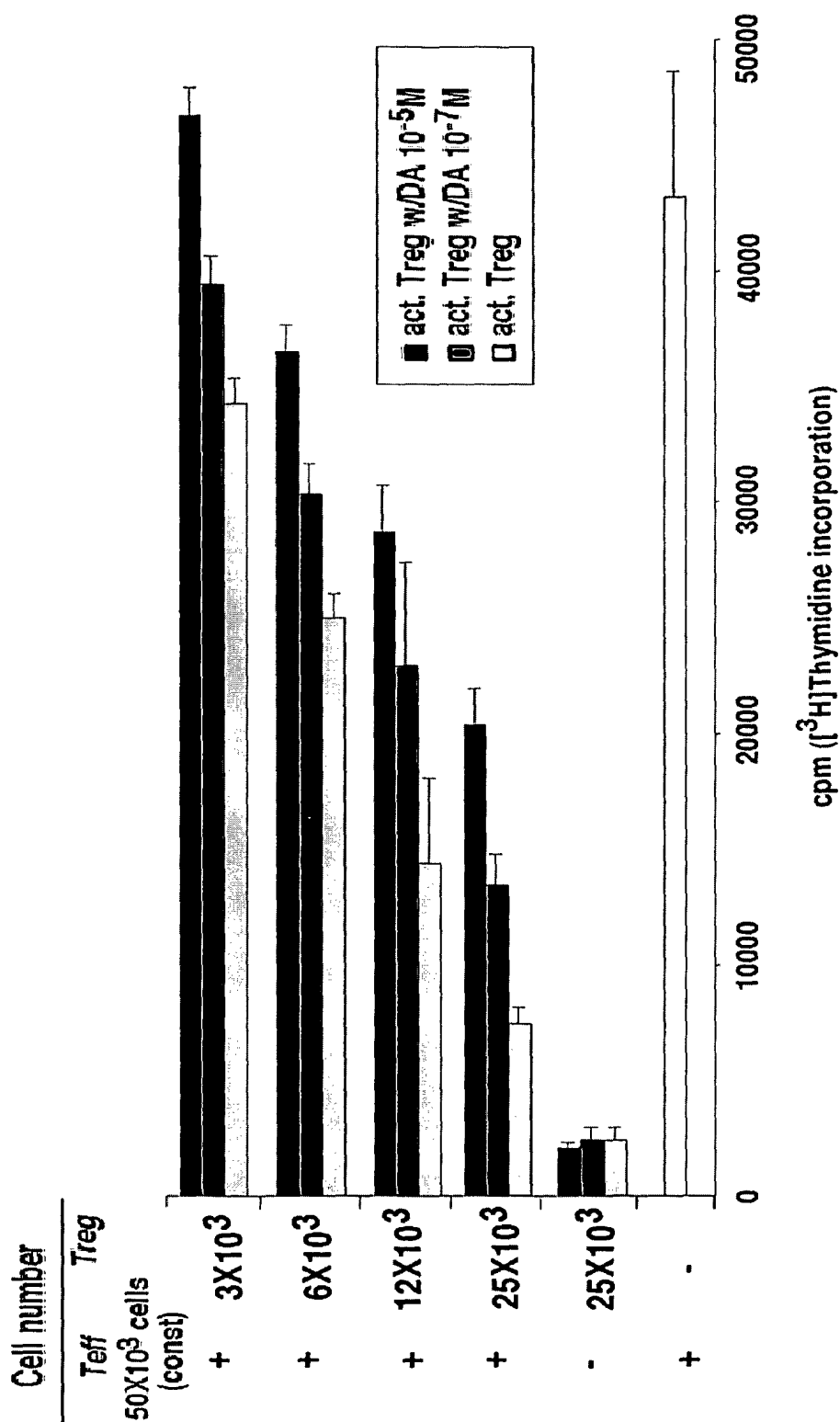


Fig. 5a

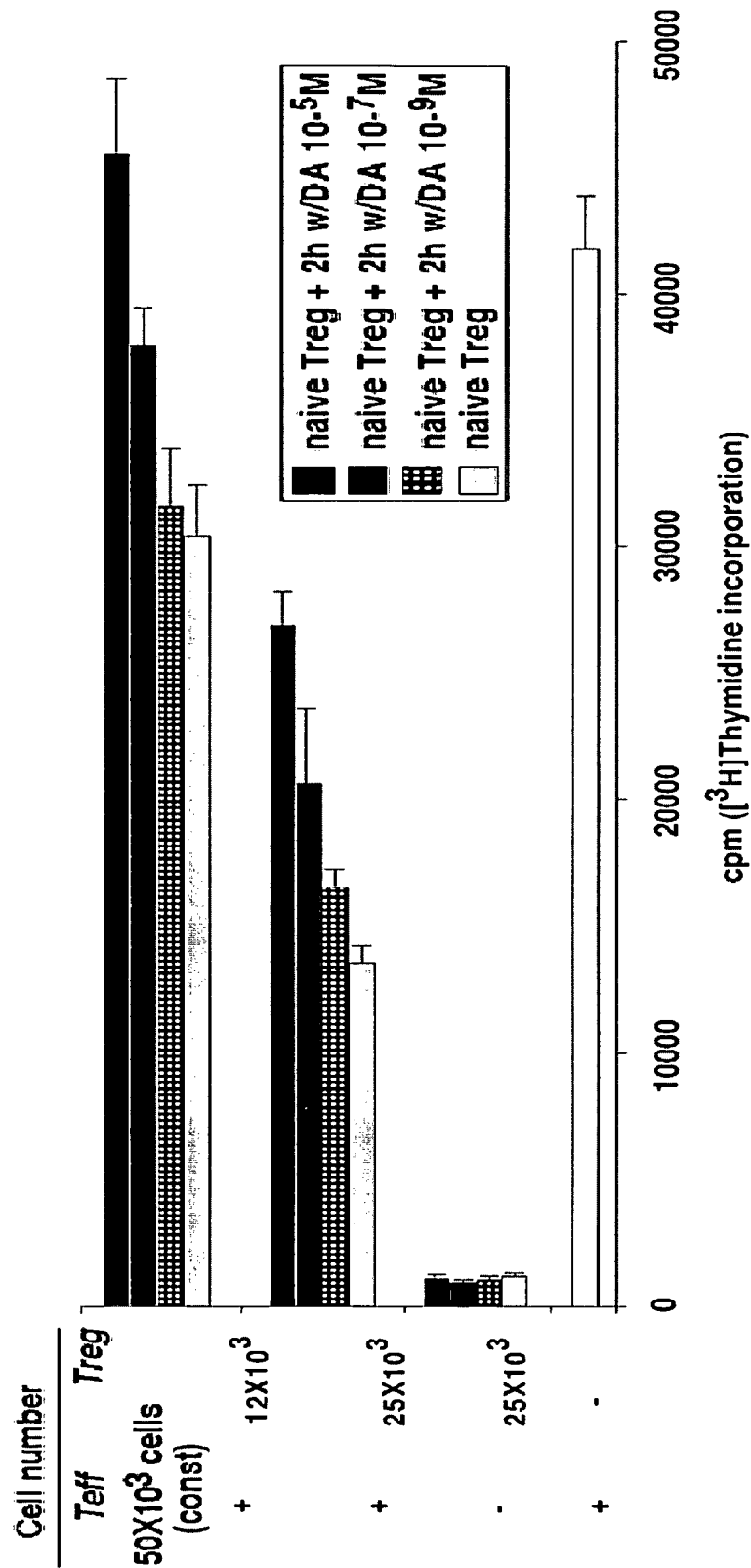


Fig. 5b

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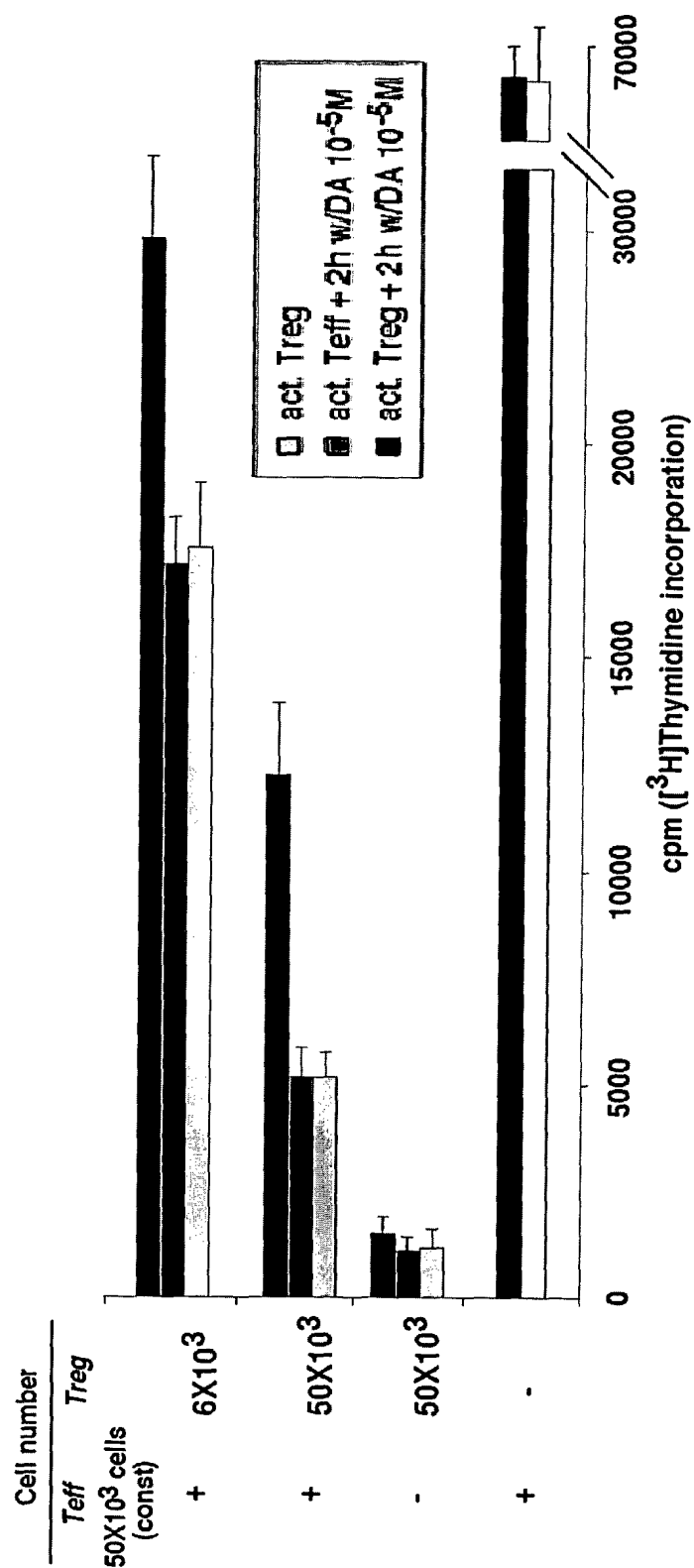


Fig. 5c

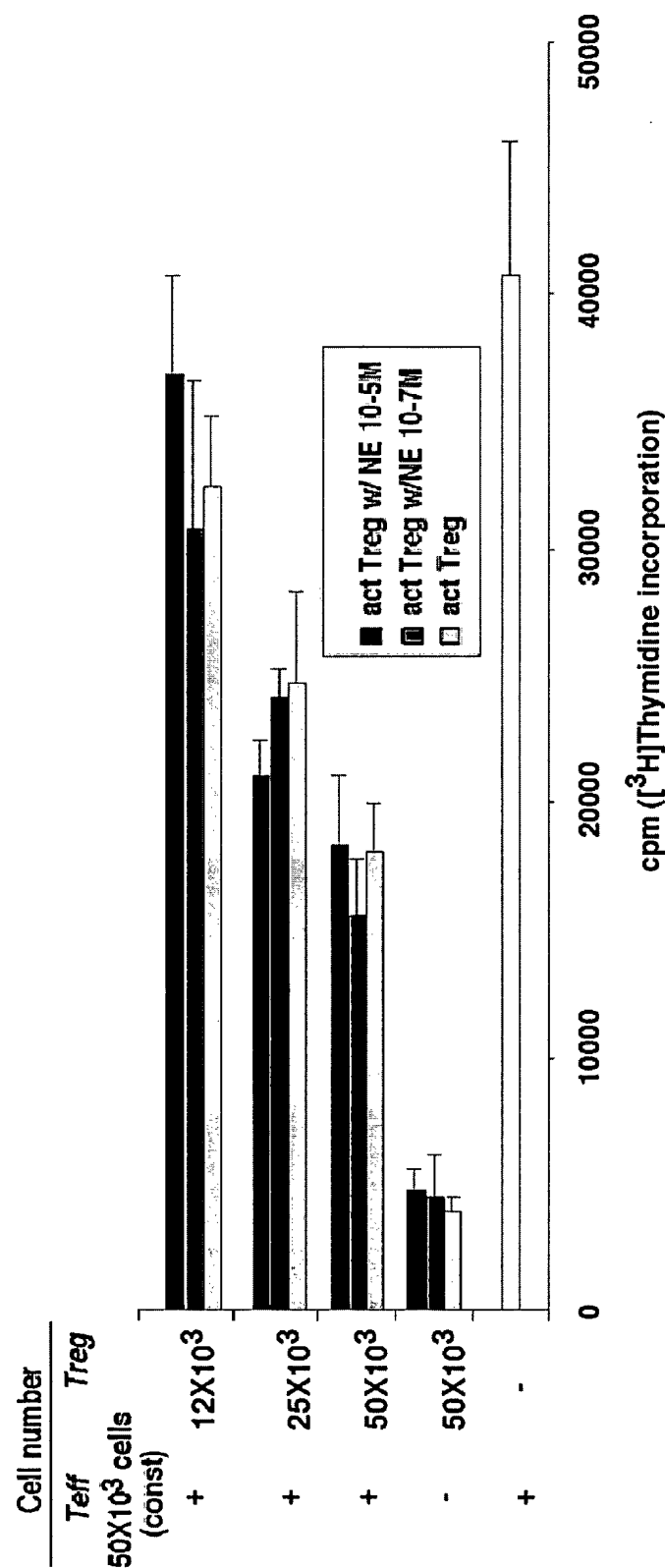


Fig. 6a

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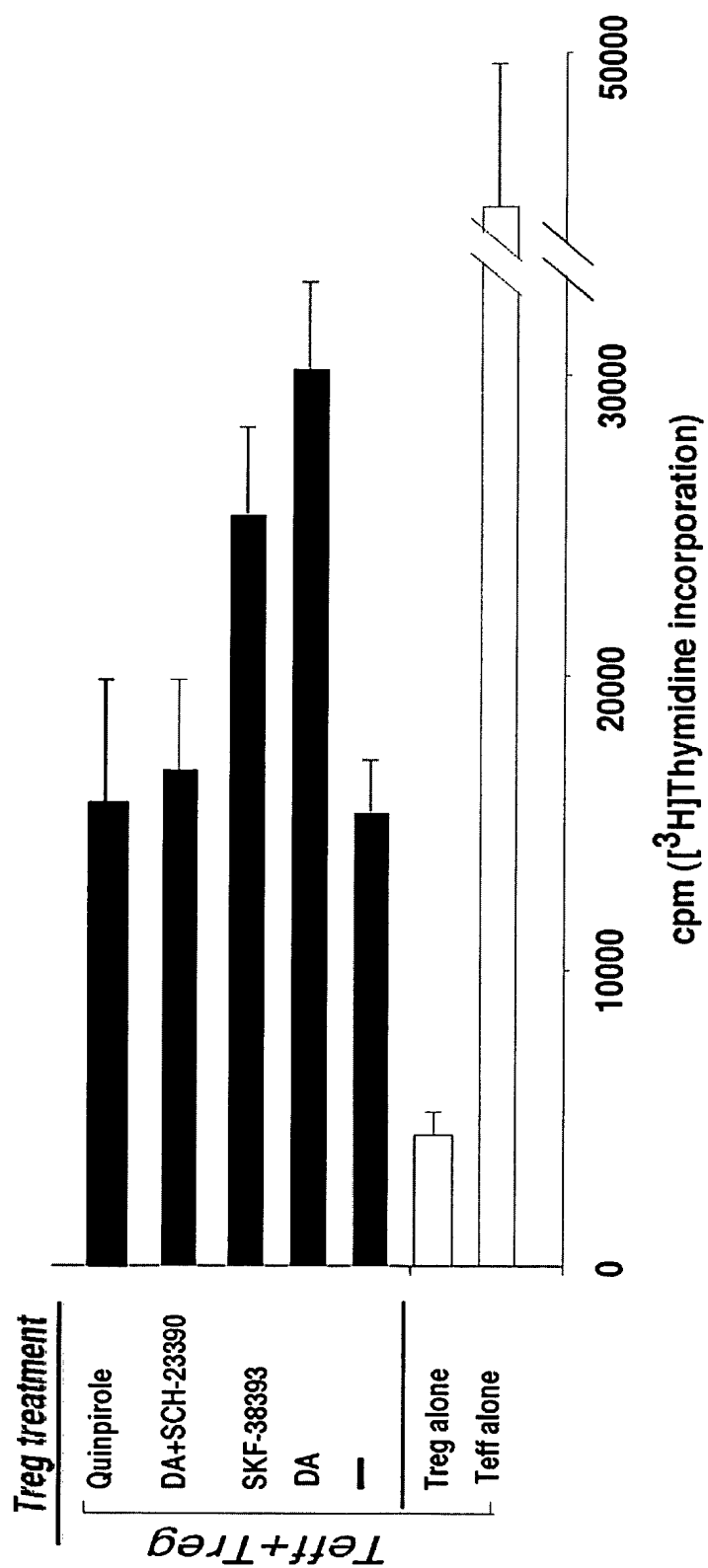


Fig. 6b

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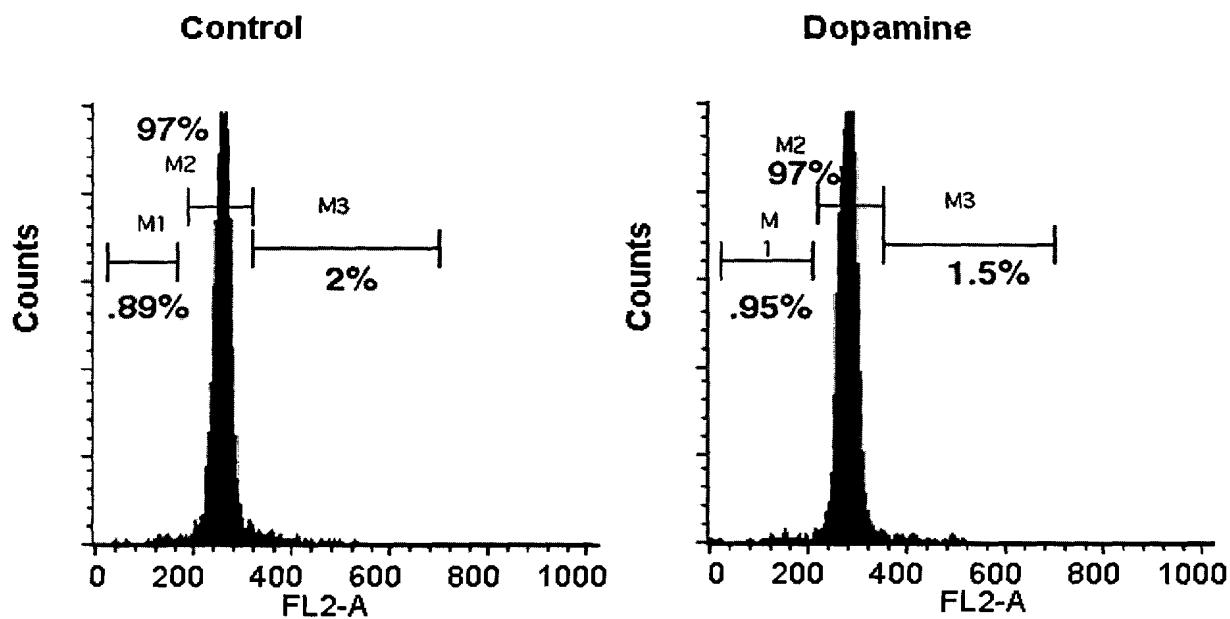


Fig. 6c

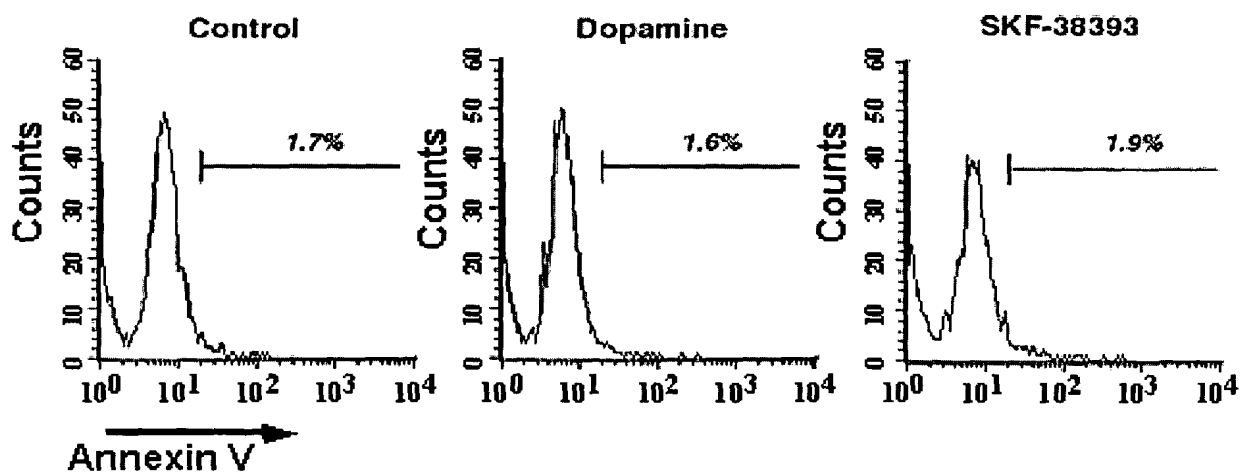


Fig. 6d

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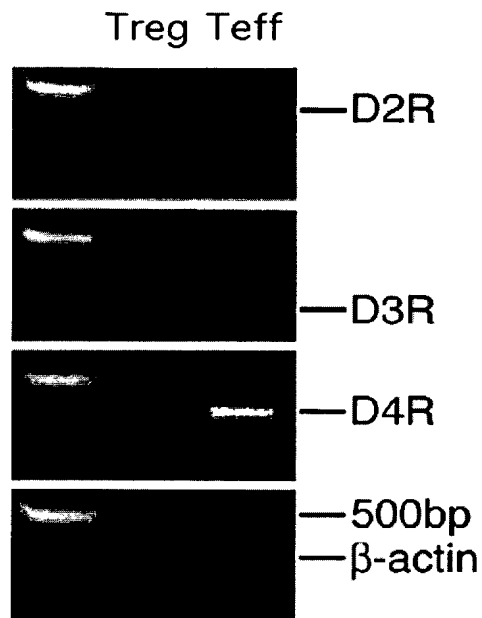


Fig. 7a

Gene	T cell sub-population	
	Treg	Teff
D1R	1.21±0.10	0.30±0.05
D5R	0.55±0.20	0.04±0.01

Fig. 7b

	<i>Levels (arbitrary units)</i>	
	D1R	D5R
Treg	60	9.5
Teff	12.5	0.7

Fig. 7c

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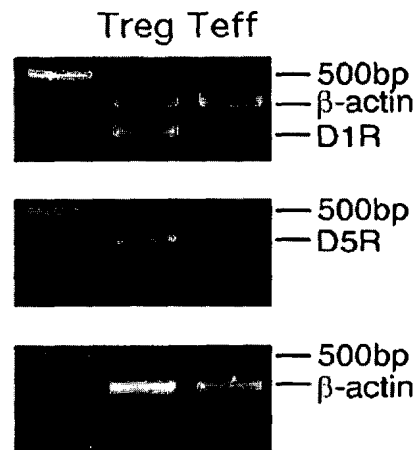


Fig. 7d

Gene	T cell sub-population	Treg	Teff
D2R		0.55 $\pm$ 0.13	0.59 $\pm$ 0.11
D3R		0.83 $\pm$ 0.24	0.79 $\pm$ 0.16
D4R		0.96 $\pm$ 0.12	1.47 $\pm$ 0.27

Fig. 7e

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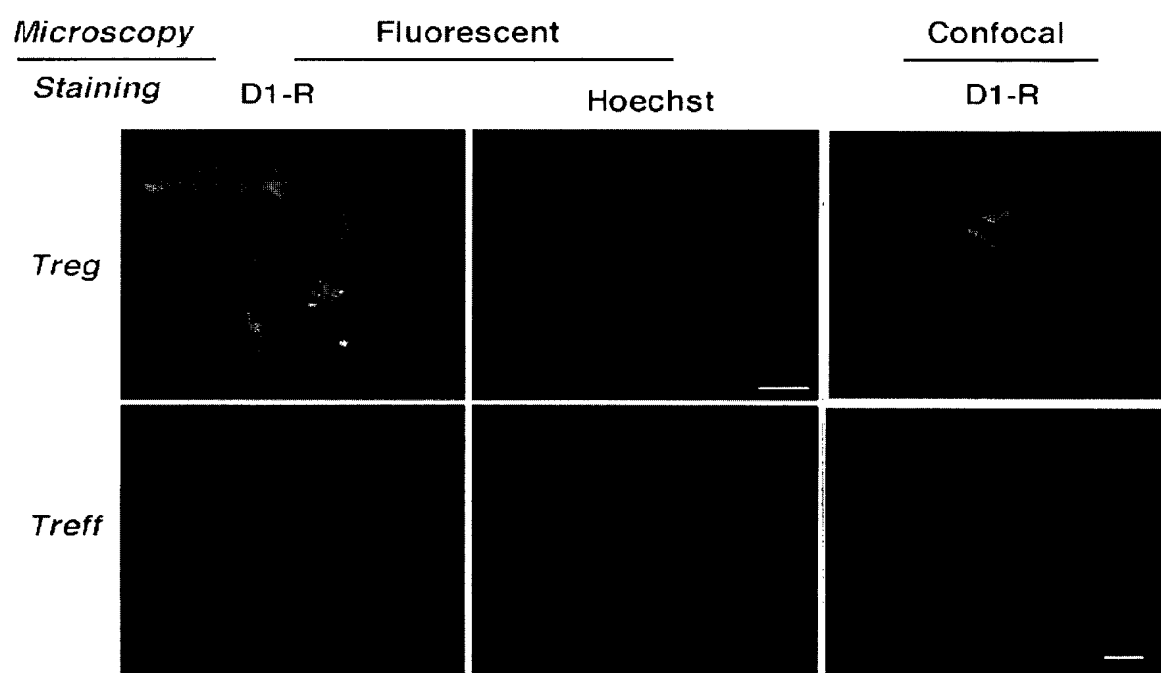


Fig. 7f

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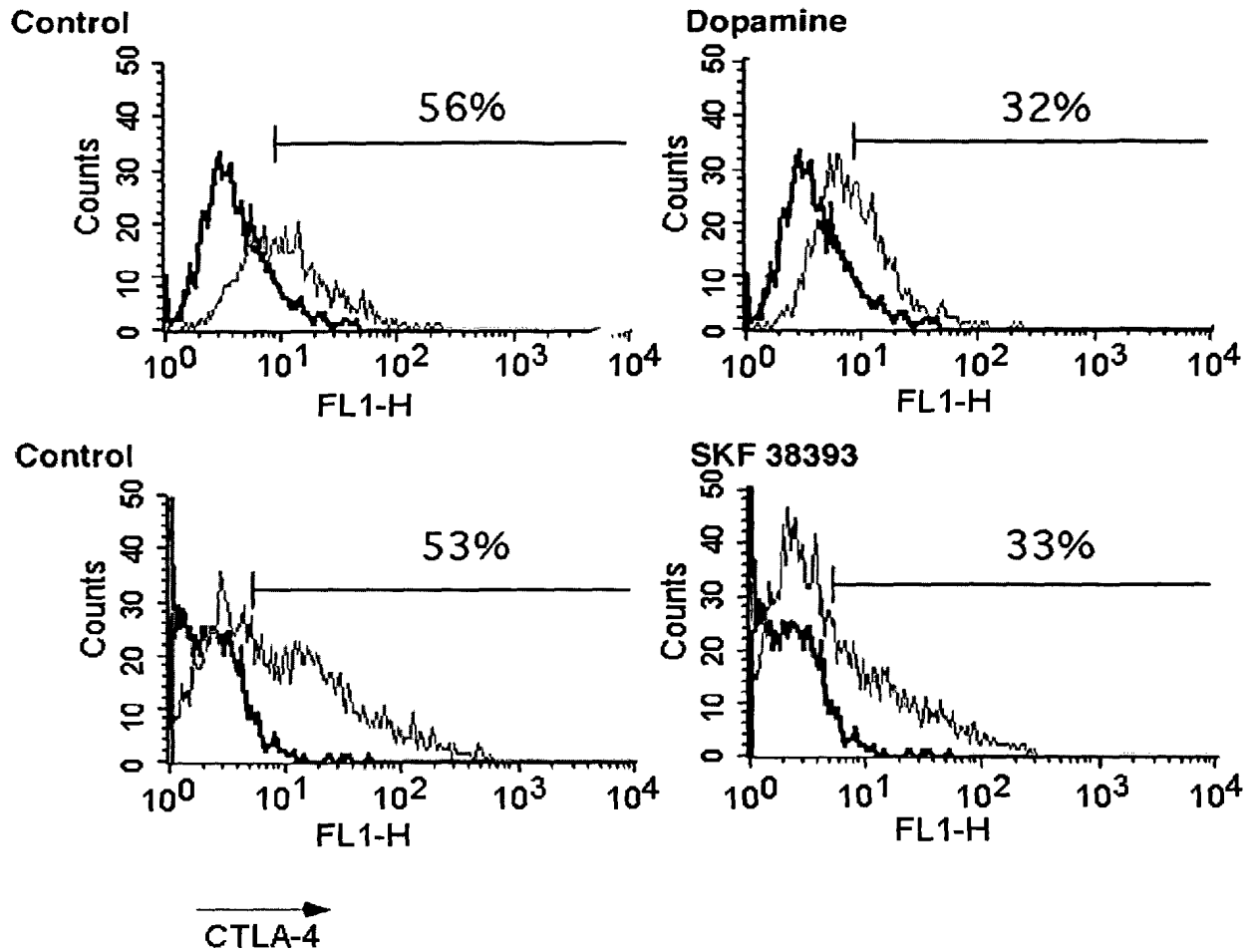


Fig. 8a

Time after DA exposure (h)	DA in culture	
	-	+
IL-10 concentration (pg/ml)		
24	523 ± 101	210 ± 32 **
48	1526 ± 105	844 ± 113 **
72	2557 ± 200	1951 ± 433 *

Fig. 8b

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CD4 sub-population \ DA in culture	-	+
	IL-2 concentration (pg/ml)	
Treg	$1.2 \pm 0.58$	$0.95 \pm 0.48$
Teff	$280 \pm 9.40$	$279 \pm 12.6$

Fig. 8c

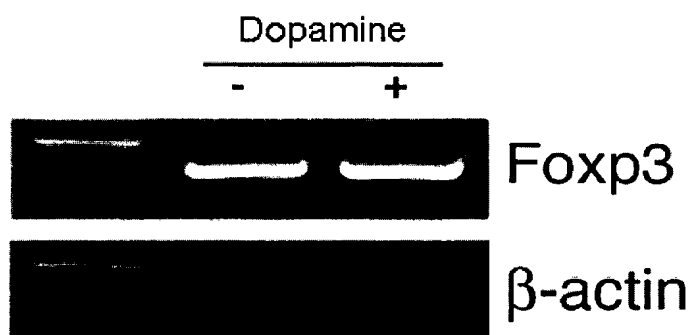


Fig. 8d

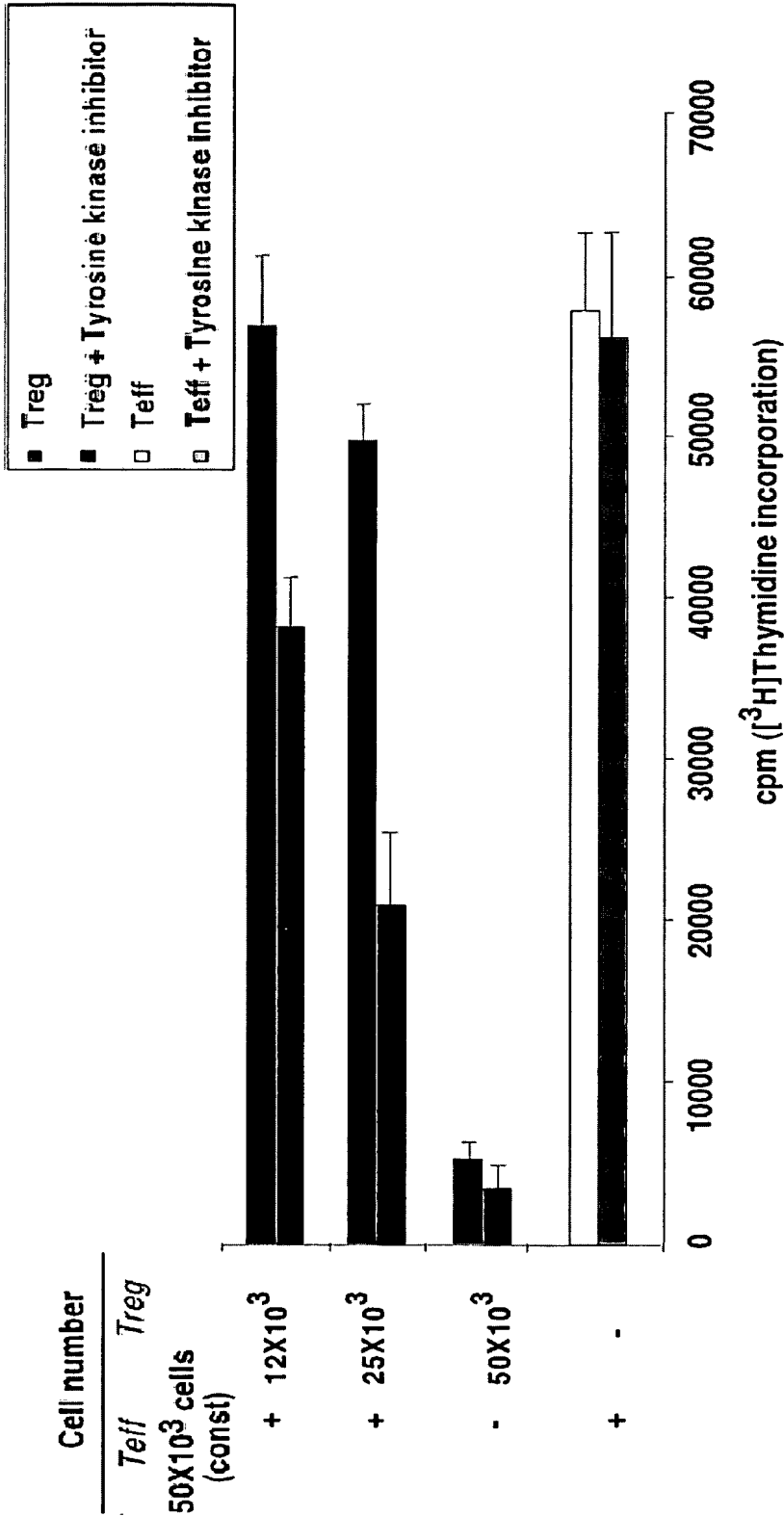


Fig. 9a

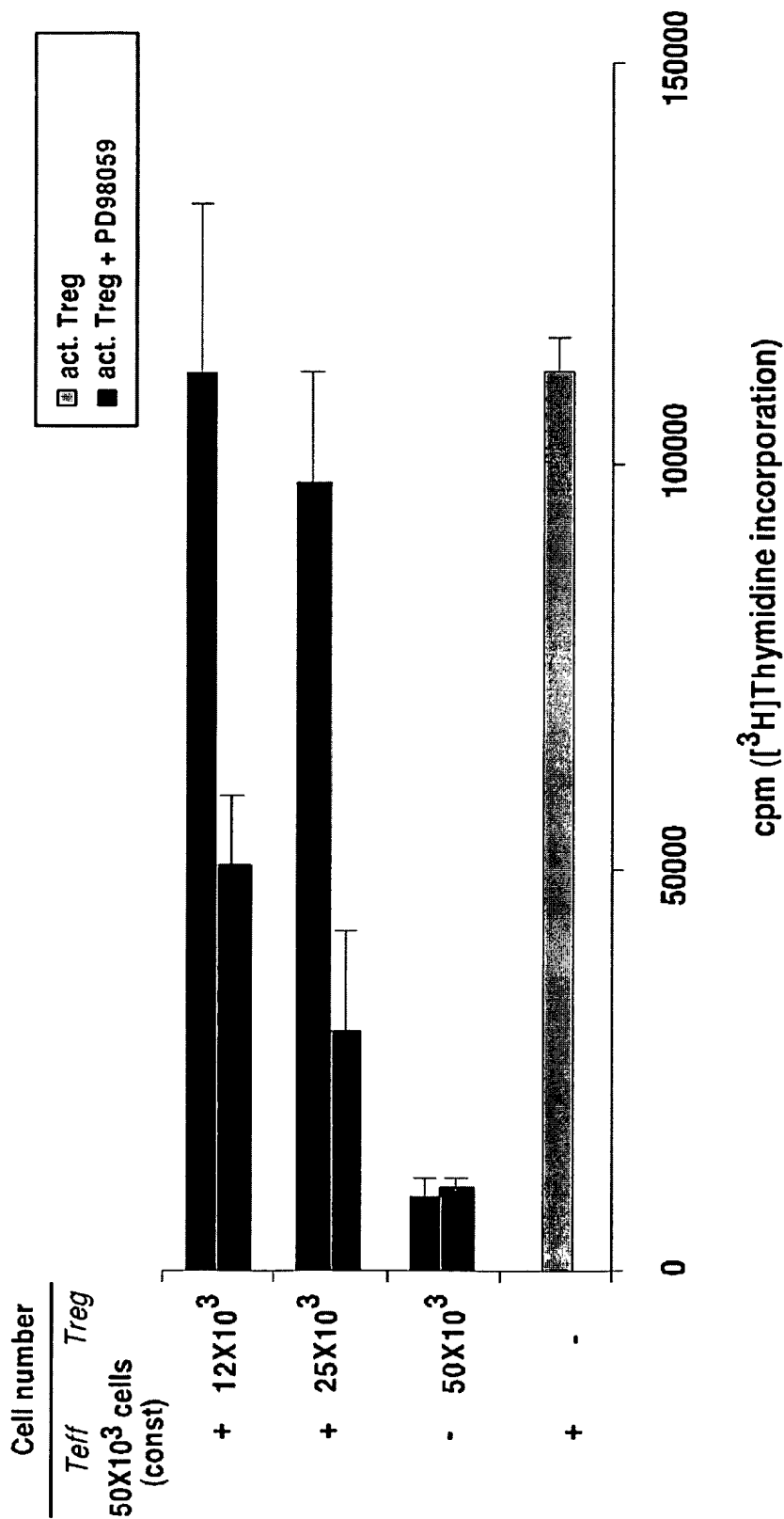


Fig. 9b

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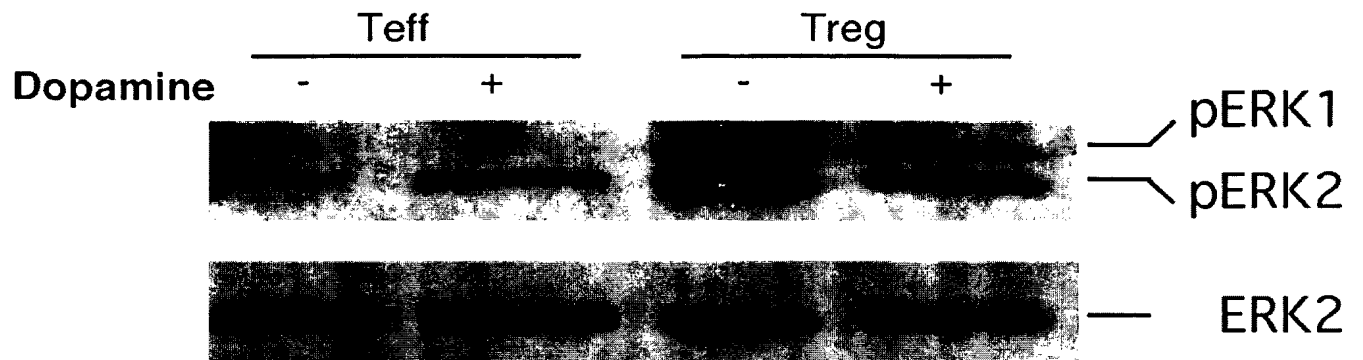


Fig. 10a

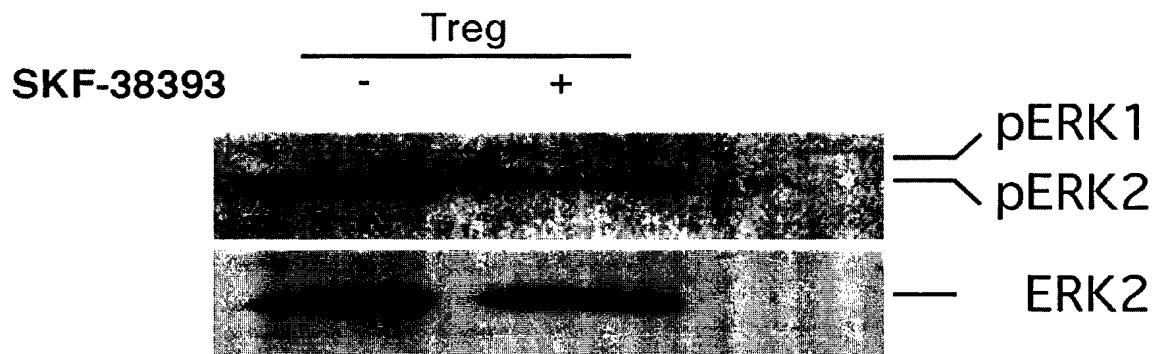


Fig. 10b

<i>Treatment</i>	Teff		Treg	
	-	+	-	+
Dopamine	0.35±0.10	0.48±0.10	3.52±0.60	1.11±0.32
SKF-38393	N/V	N/V	0.72±0.04	0.35±0.05

Fig. 10c

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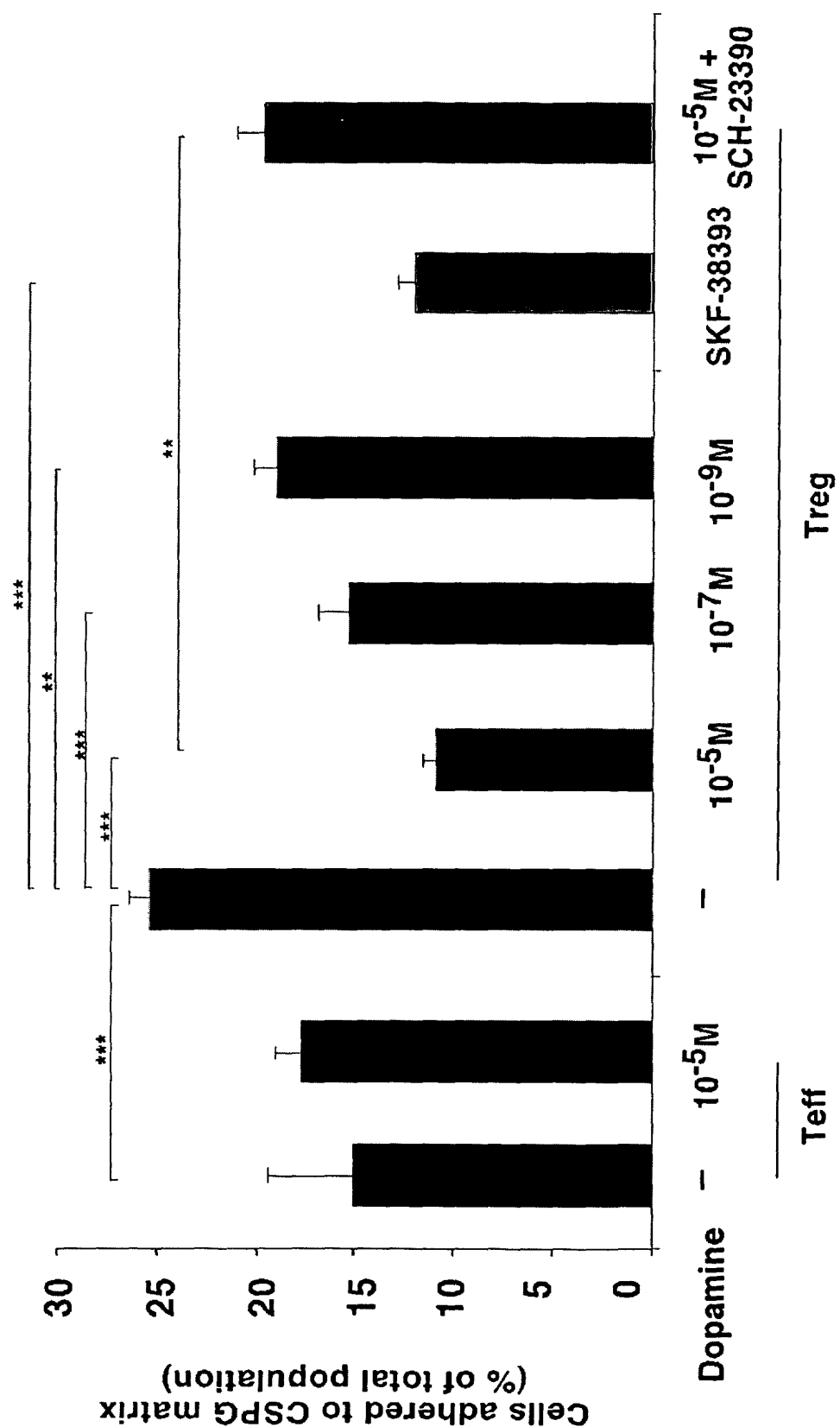


Fig. 11a

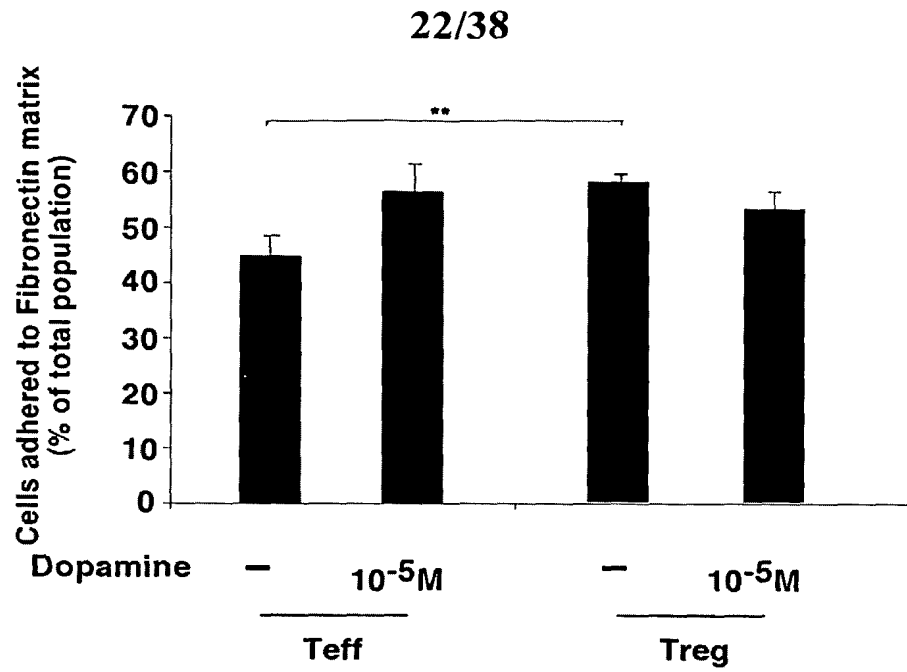


Fig. 11b

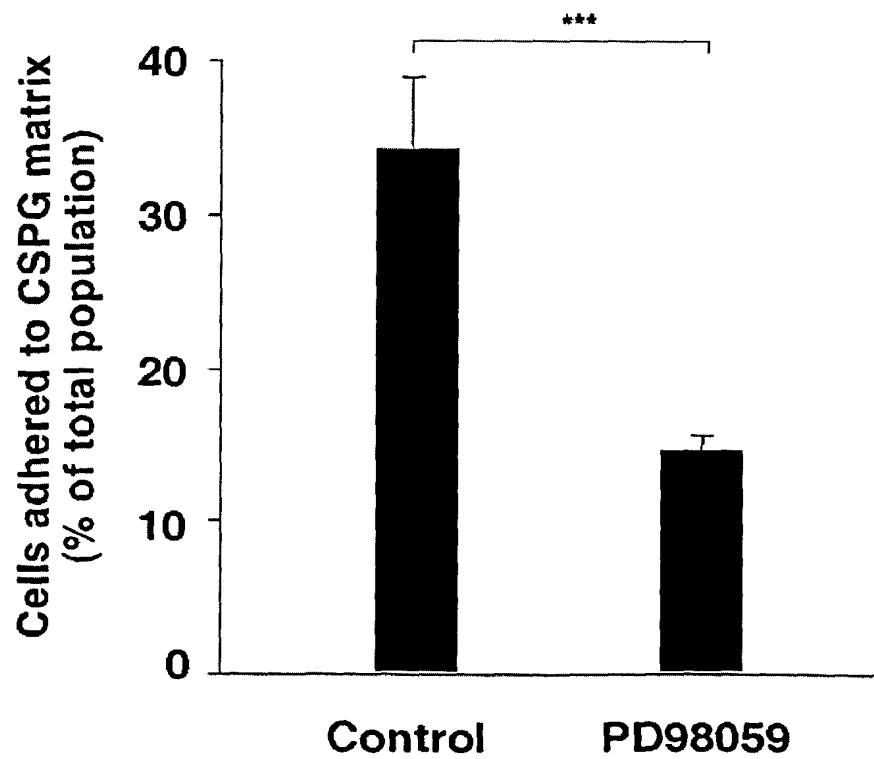


Fig. 11c

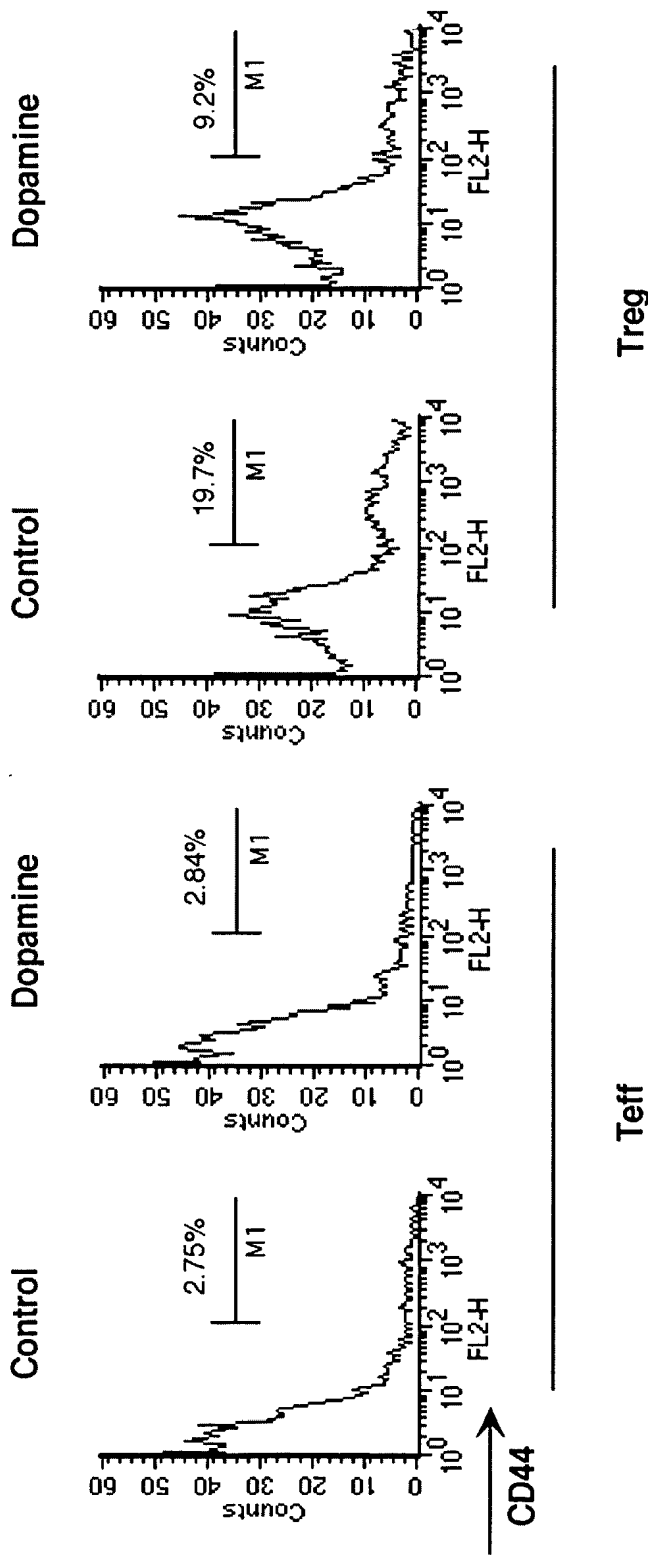


Fig. 11d

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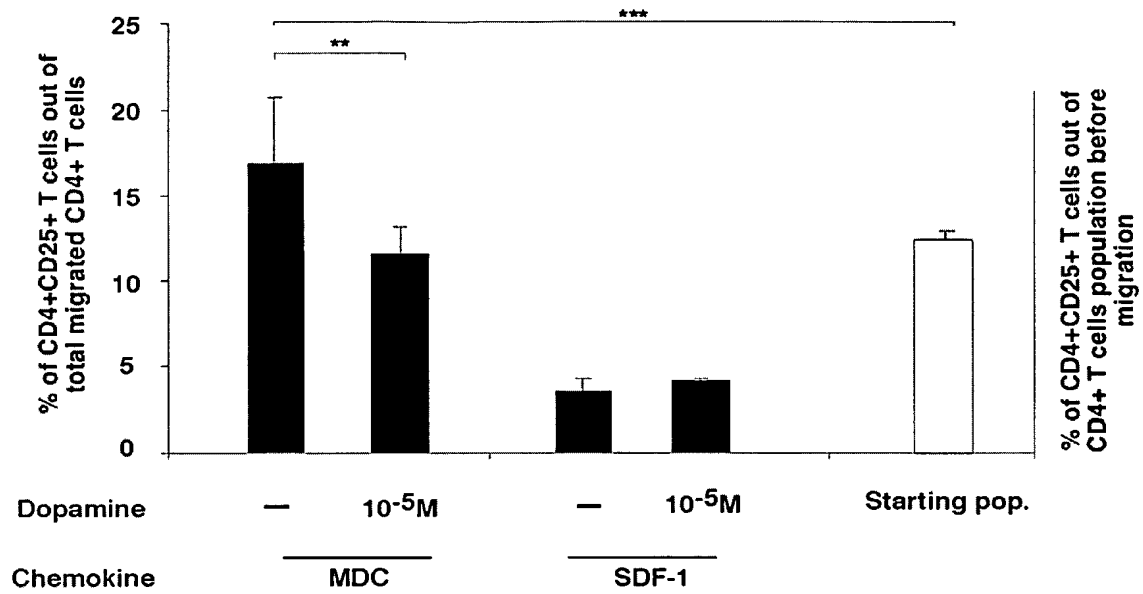


Fig. 12a

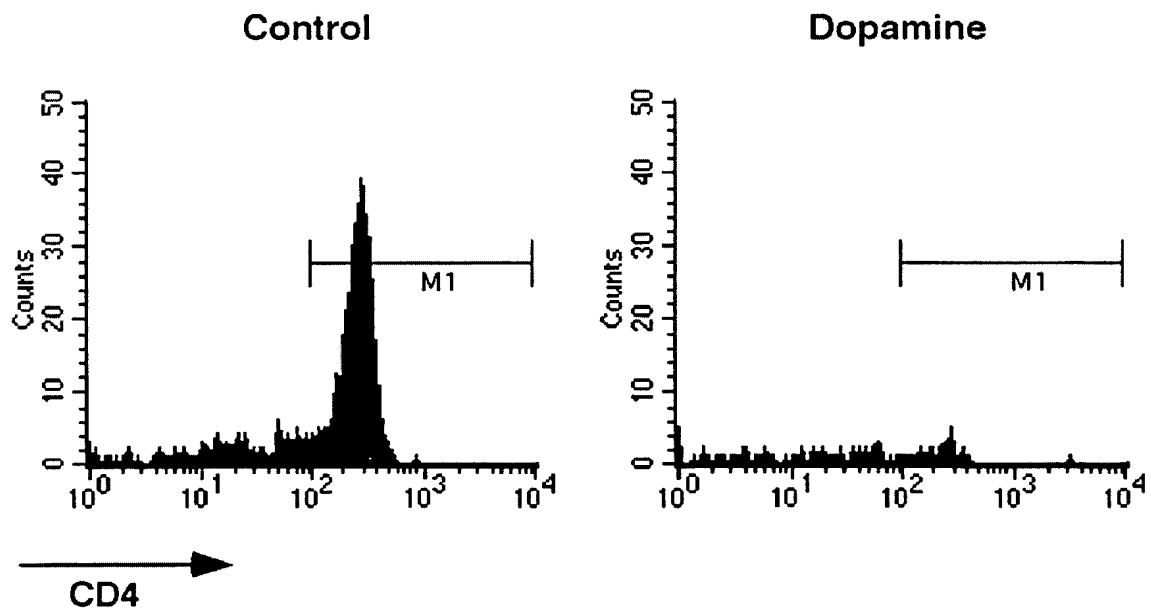


Fig. 12b

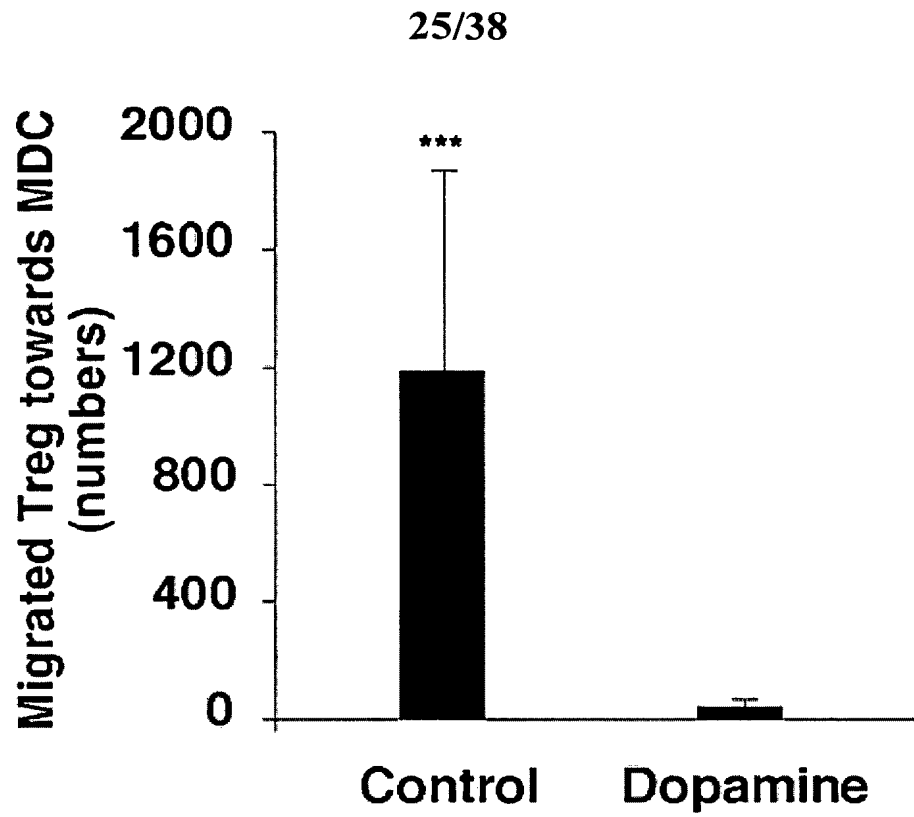


Fig. 12c

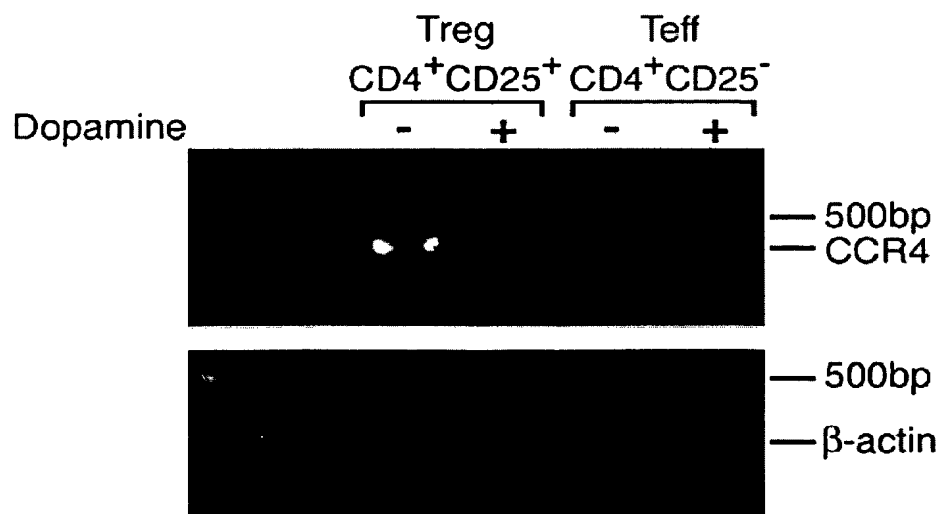


Fig. 12d

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Gene \ DA	Treg		Teff	
	-	+	-	+
CCR4	1.7±0.11	0.81±0.12	0.35±0.10	0.38±0.08

Fig. 12e

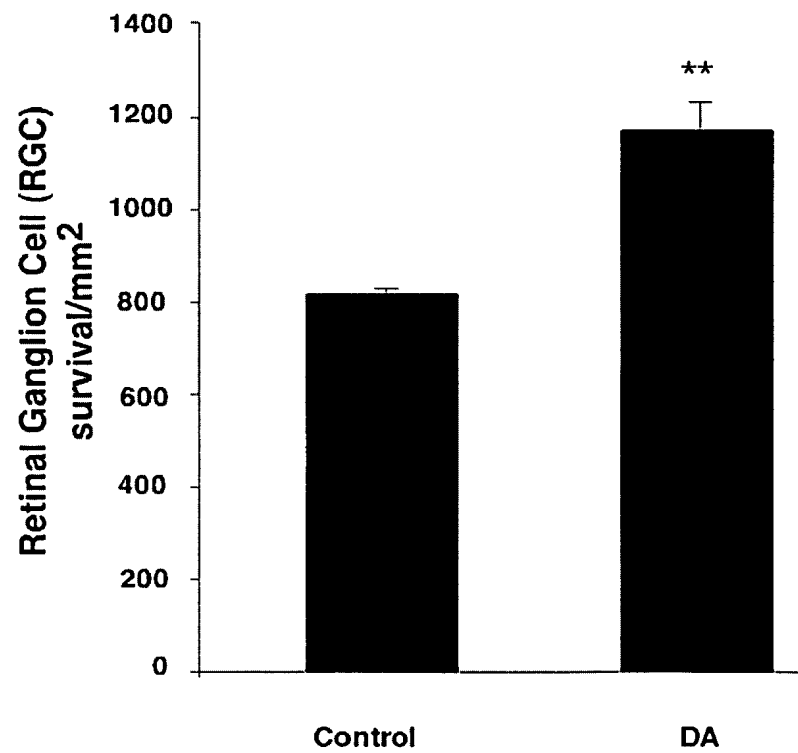


Fig. 13a

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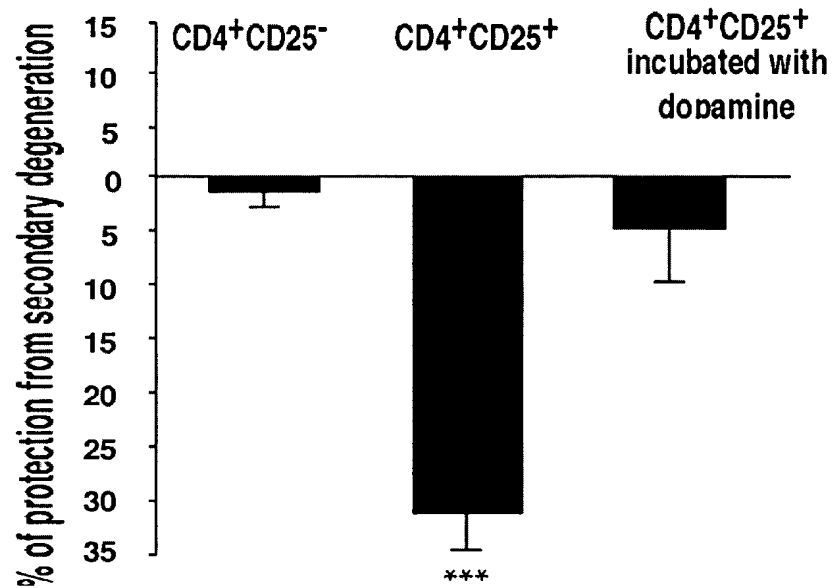


Fig. 13b

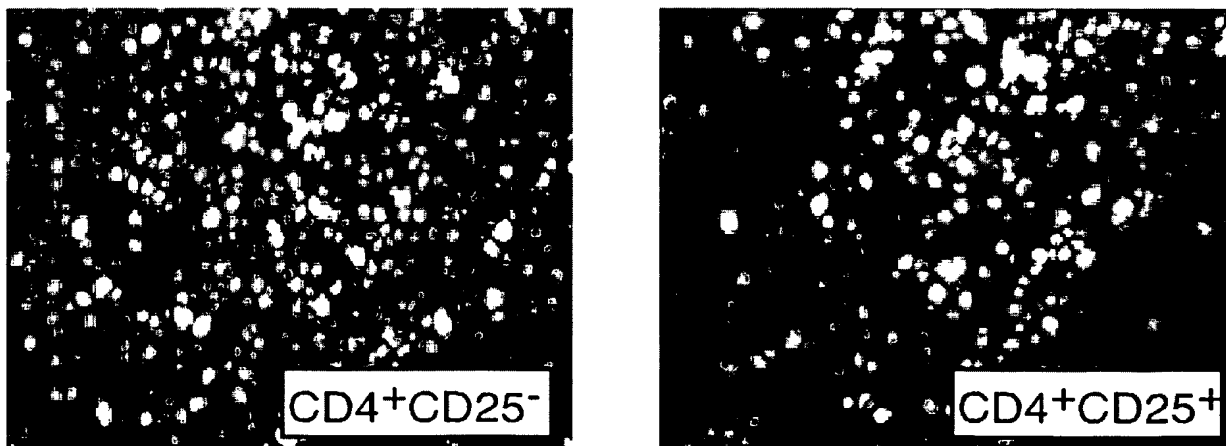


Fig. 13c

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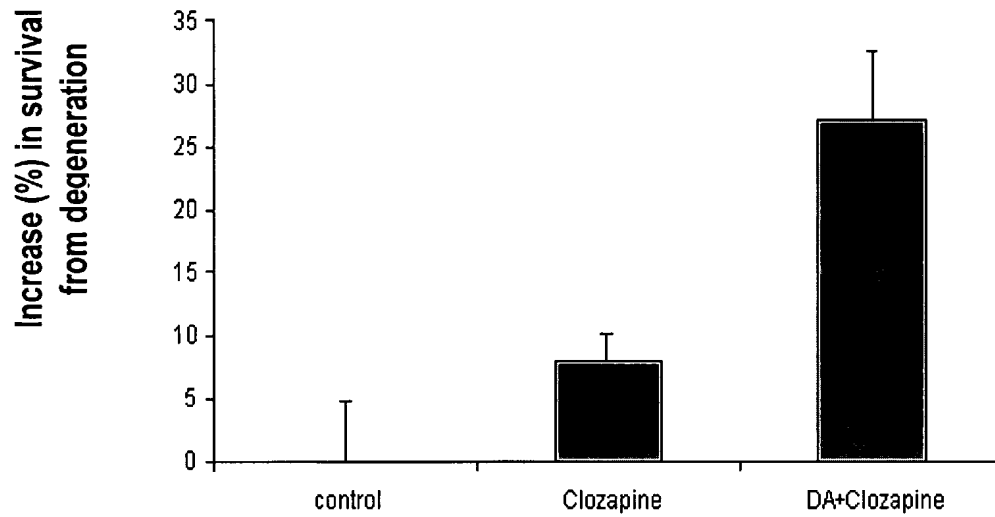


Fig. 14

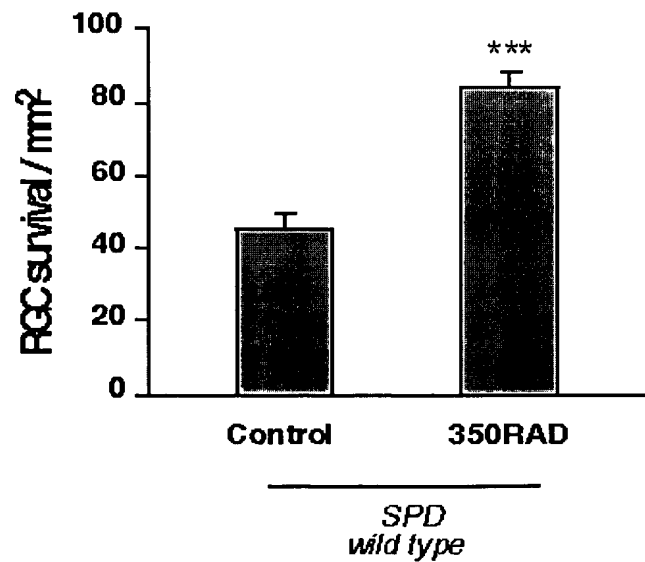


Fig. 15a

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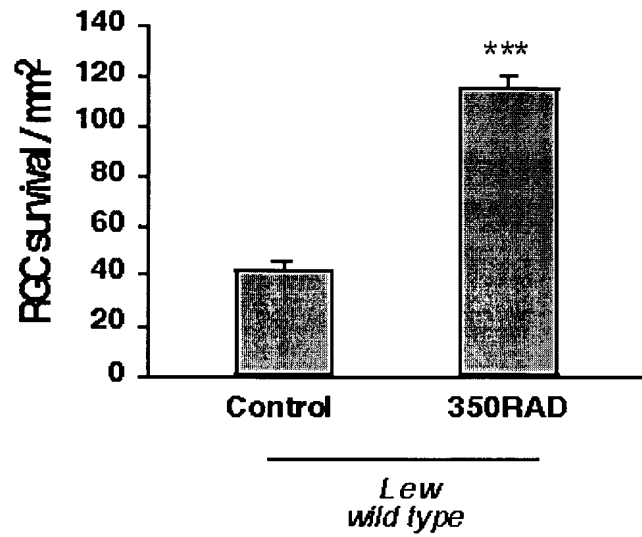


Fig. 15b

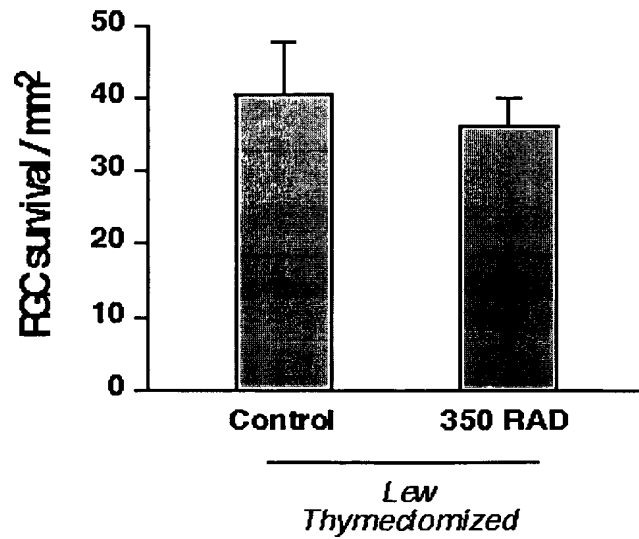


Fig. 15c

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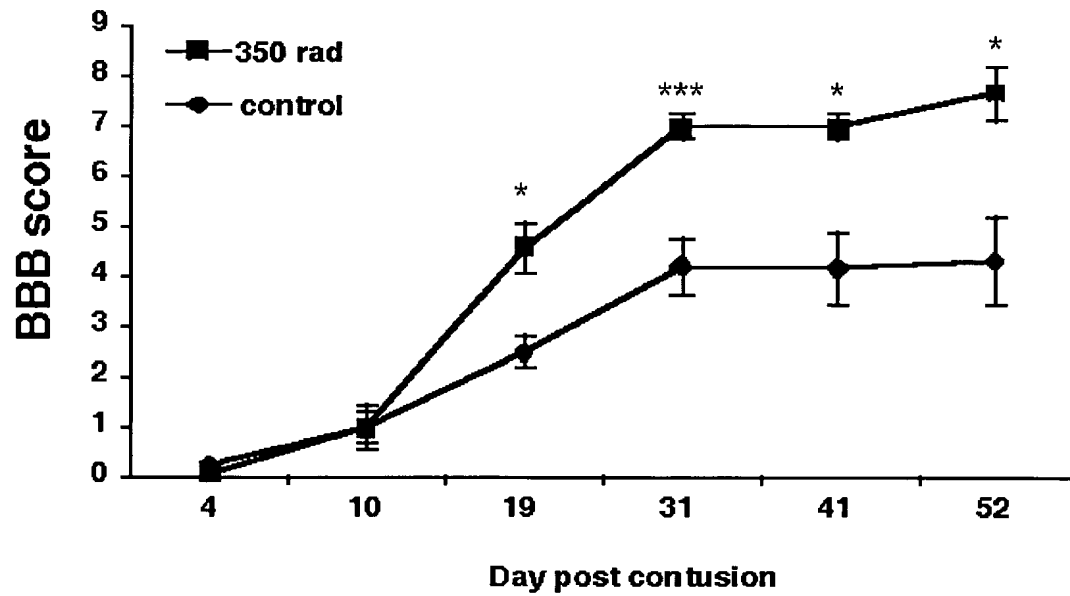


Fig. 16

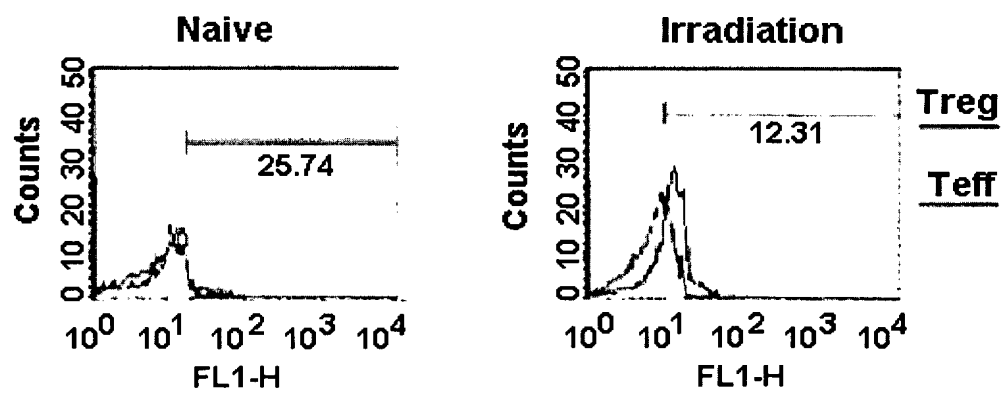


Fig. 17a

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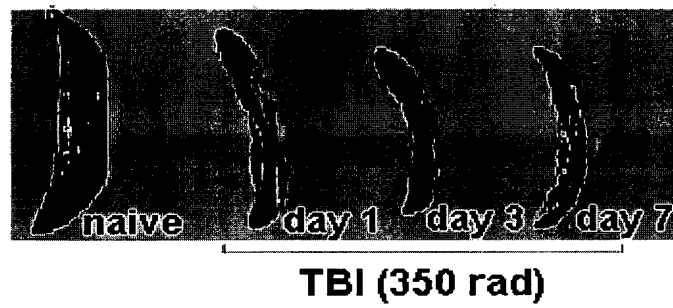


Fig. 17b

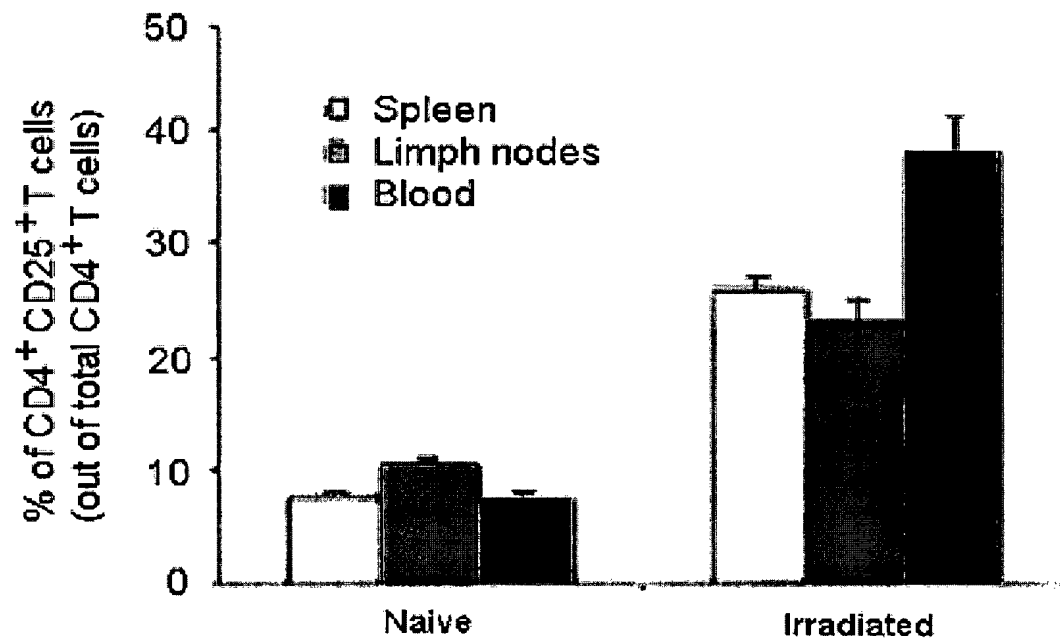


Fig. 17c

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Fig. 17d

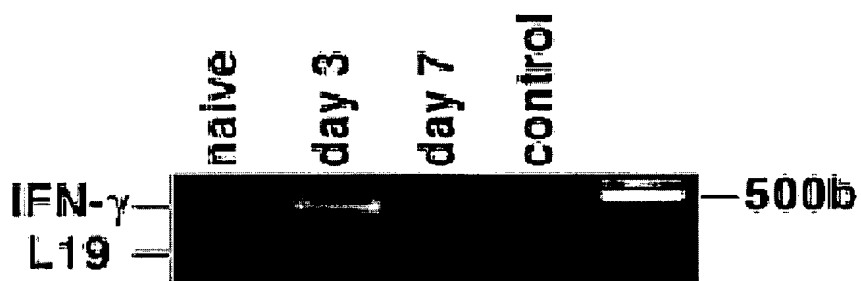


Fig. 17e

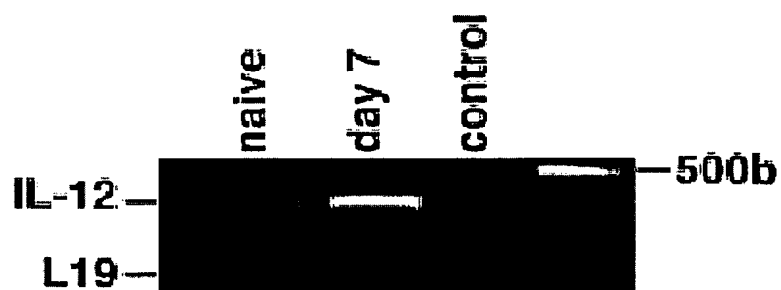


Fig. 17f

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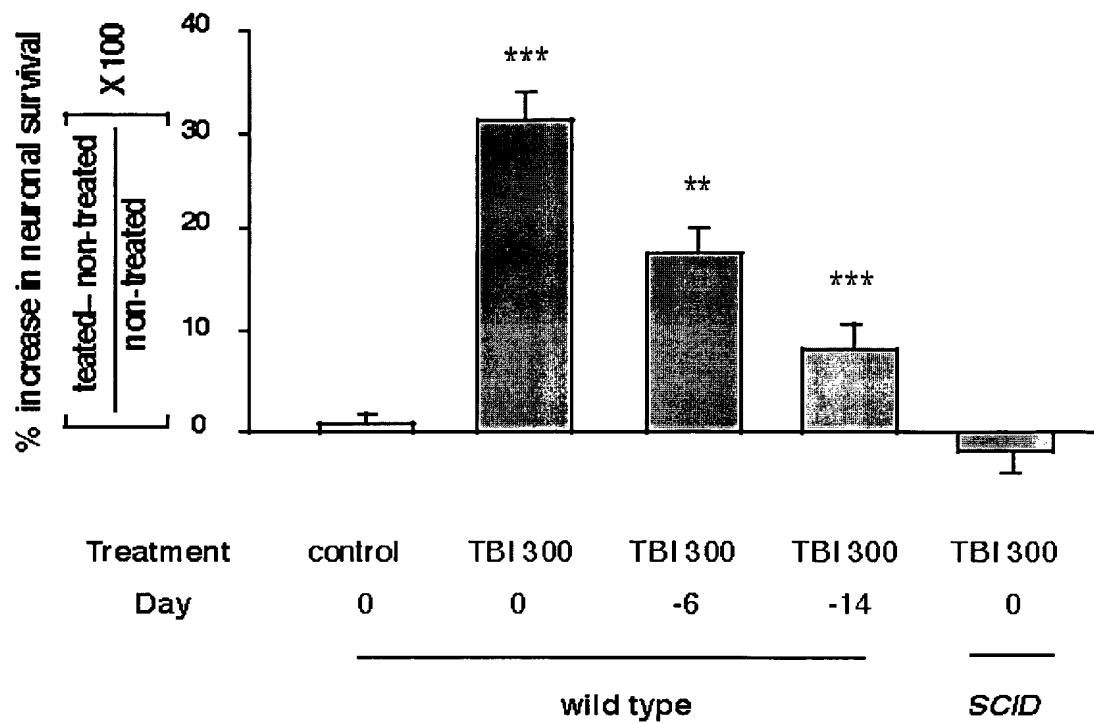


Fig. 18a

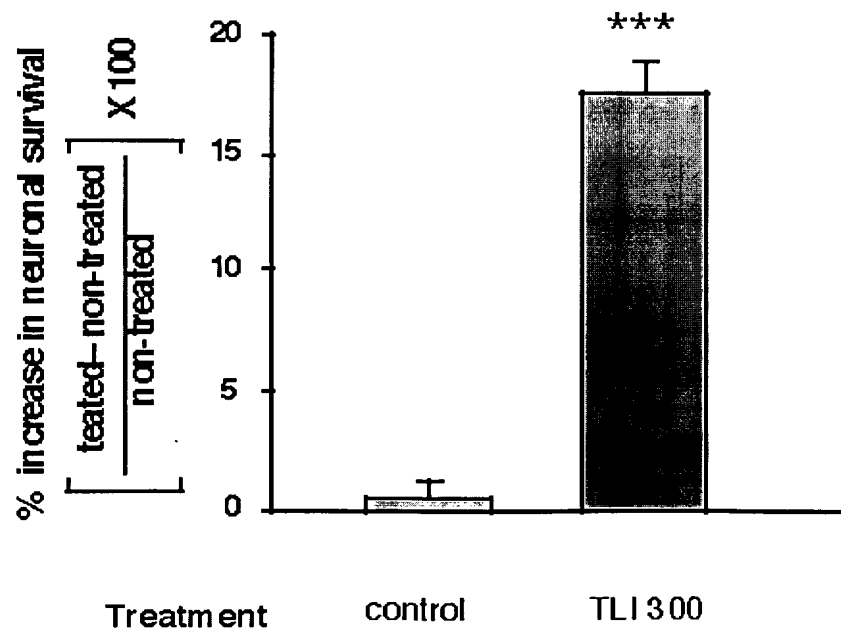


Fig. 18b

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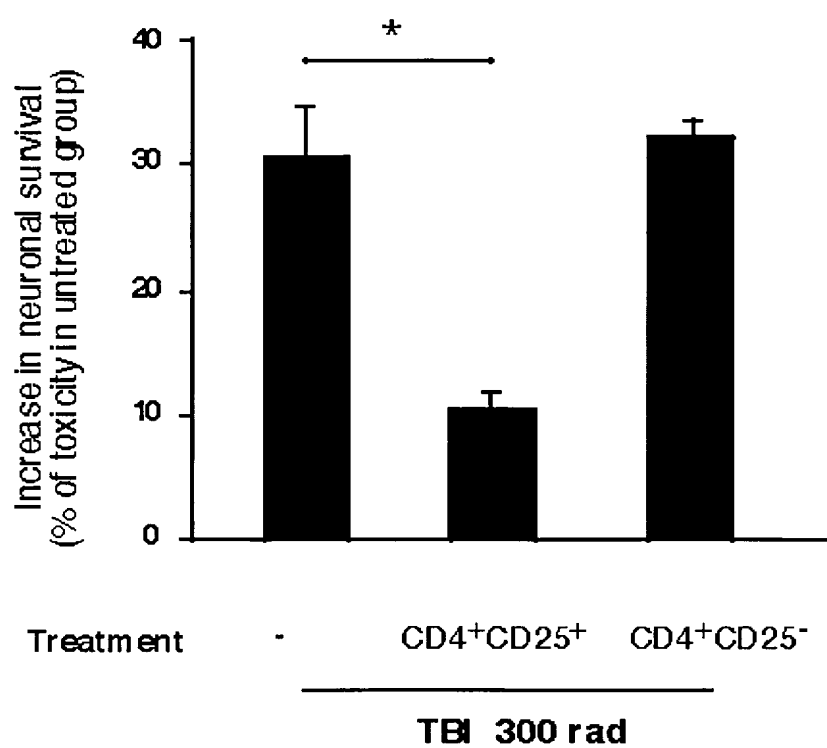


Fig. 18c

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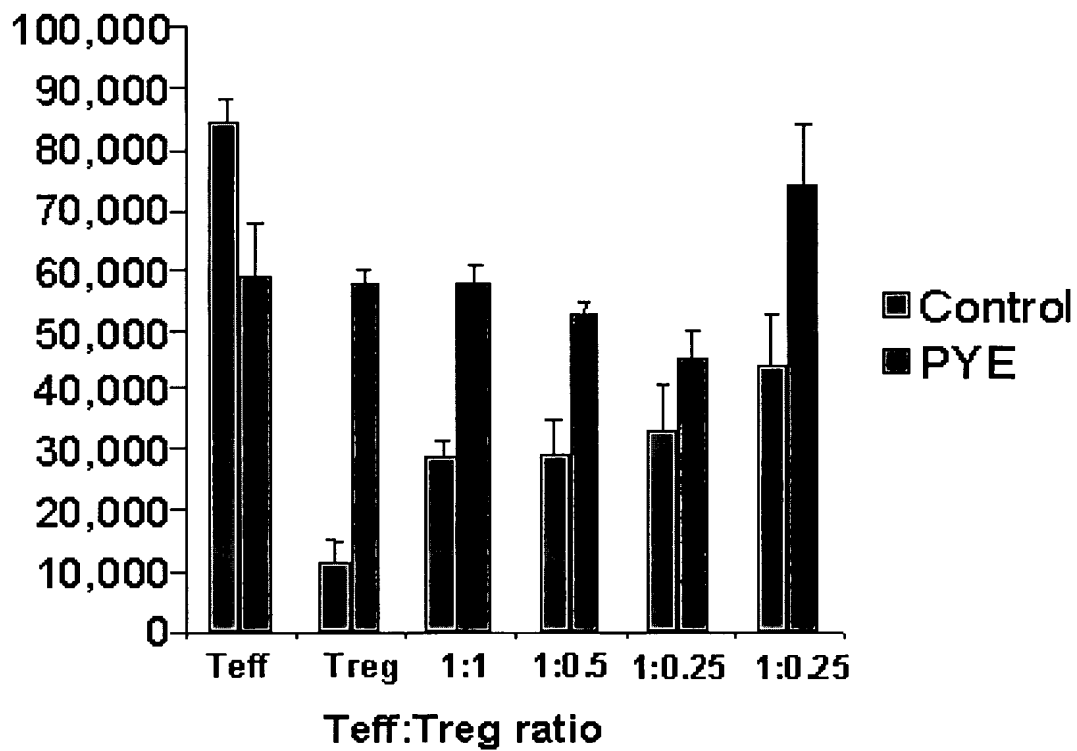


Fig. 19a

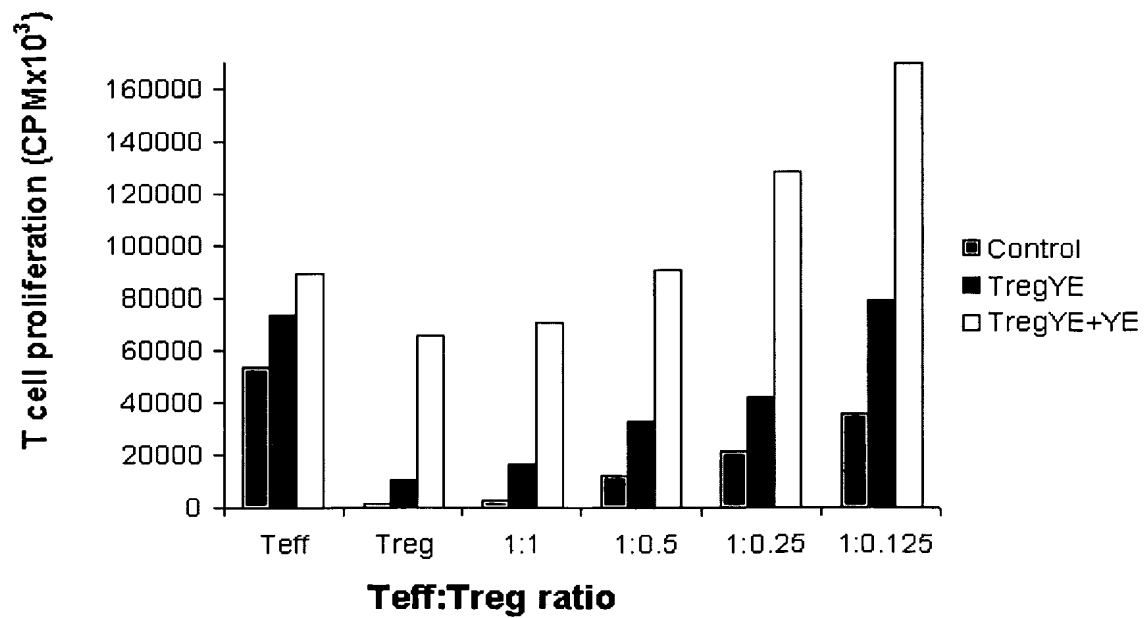


Fig. 19b

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Fig. 20a

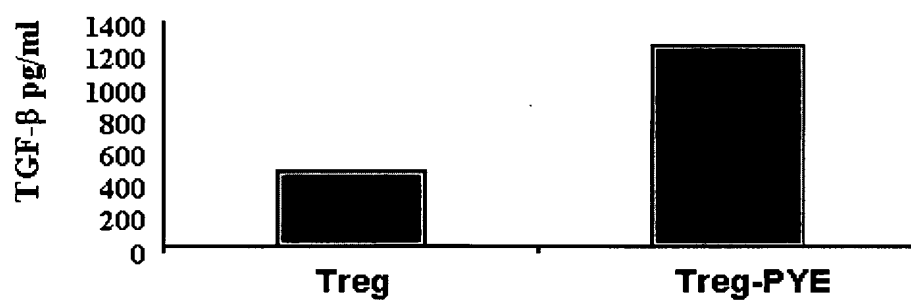


Fig. 20b

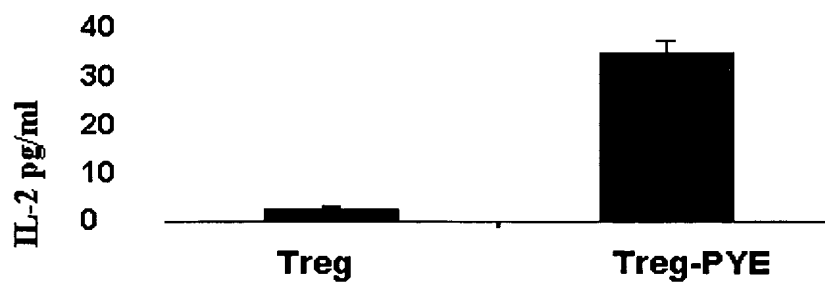
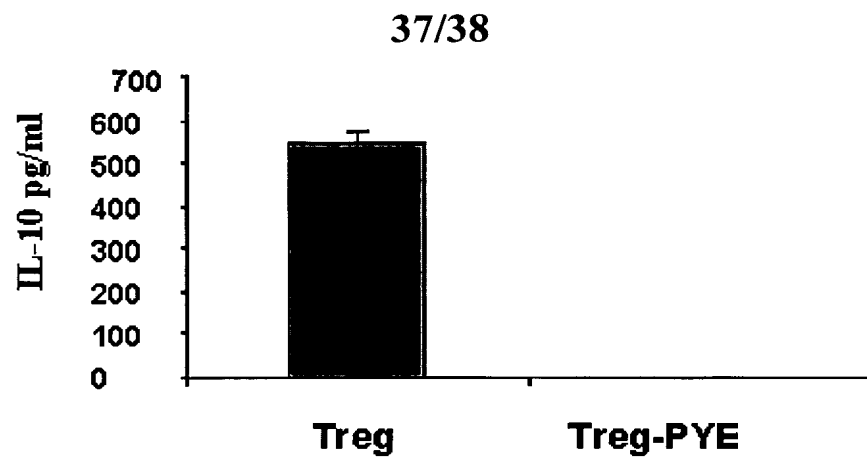
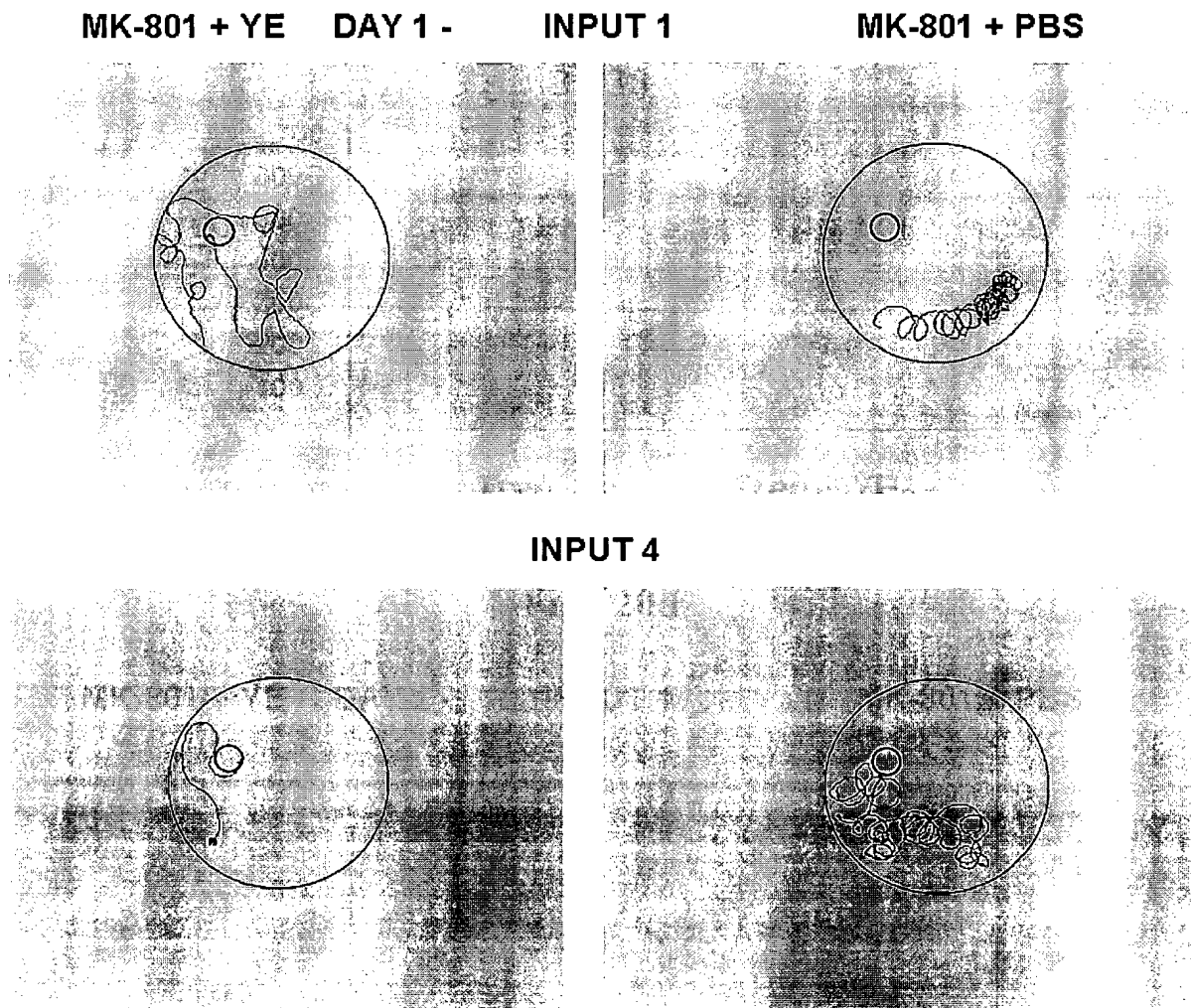


Fig. 20c

**Fig. 20d****Fig. 21**

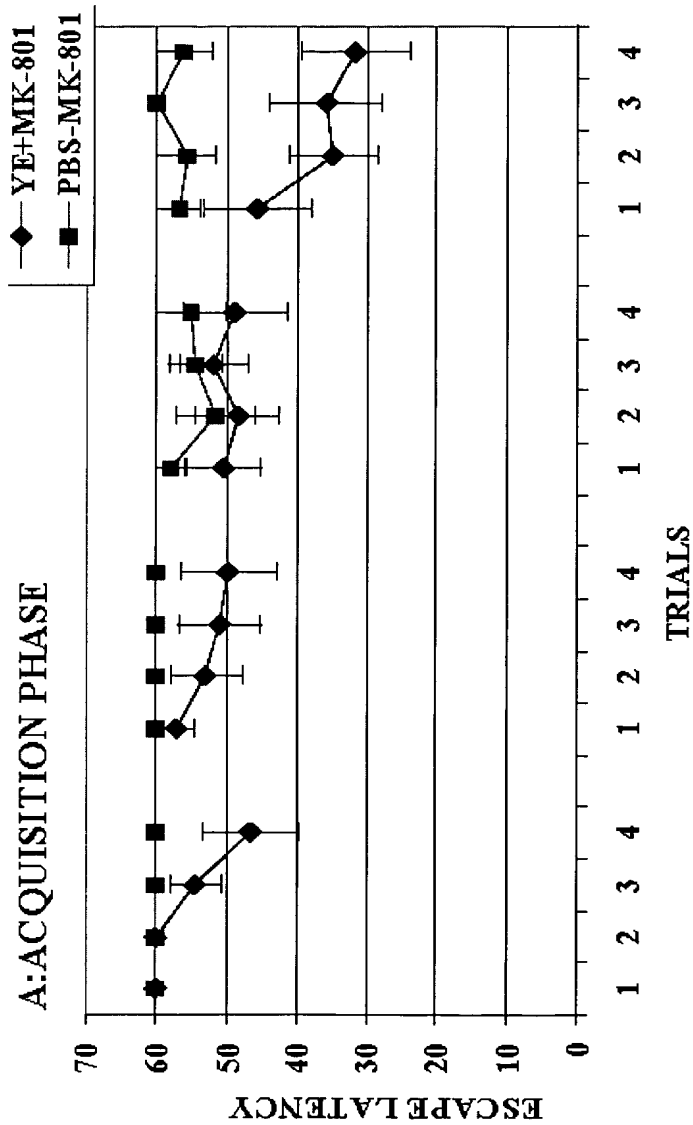


Fig. 22

## SEQUENCE LISTING

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Kipnis, Jonathan

<120> COMPOSITIONS AND METHODS FOR TREATMENT OF PSYCHIATRIC DISORDERS

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